# PERICYTES ARE MORE THAN MSCS:
# A COMPARISON OF THREE CELL POPULATIONS

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**NATIONAL UNIVERSITY OF SINGAPORE**

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# PERICYTES ARE MORE THAN MSCS:

# A FUNCTIONAL COMPARISON OF THREE CELL POPULATIONS

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# *Declaration*

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Wang Yingting

27 May 2012

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## *Summary*

Pericytes are cells located inside the basement membrane of blood vessels. They play an essential role in angiogenesis as well as in vessel maintenance and stabilization. Recently it has been found that pericytes from various tissues demonstrated features of mesenchymal stem cells (MSCs). It has thus been proposed that some pericytes may be MSCs residing in a perivascular niche and serving as a progenitor reserve for tissue regeneration in response to injury by differentiation into other lineages. In this study, we hypothesized that apart from possessing MSC-like characteristics, pericytes further possess angiogenic functions that conventional MSC cannot substitute for. To verify if commercially purchased placenta pericytes are truly MSC-like, the expression of pericytes, MSCs, and fibroblasts (negative control) of the MSC antigen profile was compared. It was found that the marker expressions profile of all three cell types all fulfilled the marker panel required of MSCs. Interestingly, CD146, the surface marker which is used to isolate pericytes from various tissues, was expressed by all three cell types. To conclude, a conventional MSC marker profile is not sufficient to identify MSC. Therefore we further investigated the differentiation potential of the three cell types and found that only pericytes and MSCs were capable of adipogenesis and osteogenesis, indicating that pericytes as MSC are multipotent. Once we were able to show that pericytes behave like MSC, we posed the question if pericytes are more than just MSC. The three cell types were therefore compared for pericytic features. It was found that pericytes expressed NG2, desmin and Tie2, which are pericytic markers linked to important functions in angiogenesis that MSCs and fibroblasts do not share. As CD146 is not selective for the pericytes we propose a

new set of potential markers, which will have to be verified in the isolation of pericytes. The *in vitro* pro-angiogenic ability of pericytes, MSCs, and fibroblasts were also investigated using a Matrigel$^{TM}$ assay, and it was observed that pericytes, MSCs and fibroblasts all co-localized with endothelial cell networks. However, MSCs and fibroblasts contracted the network in a cell-ratio dependent manner. These findings suggested that pericytes are truly MSC-like cells, with additional role in angiogenesis distinct from that of MSCs.

In conclusion, the traditionally employed *in vitro* method to identify pericytes by the co-localization of cells with tubular network on Matrigel$^{TM}$ is inconclusive and not sufficient. In order to distinguish pericytes from other cells in the tube formation assay pericyte and non-pericyte standards have to be considered and the contraction of the network over time observed.

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# *List of symbols and abbreviations*

BSA: bovine serum albumin
DMEM: Dulbecco's modified Eagle medium
EC: endothelial cells
FB: fibroblasts
FBS: fetal bovine serum
FC: Flow Cytometry
HBSS: Hanks' balanced salt solution
HUVEC: human umbilical vein endothelial cells
ICC: Immunocytochemistry
MSCs: mesenchymal stem cells
PBS: phosphate buffered saline buffer
Pl-Prc: placenta pericytes
p/s: antibiotic-penicillin/streptomycin
SMC: smooth muscle cells

# *1. Background: MSCs and pericytes —interweaving identities*

Mesenchymal stem/stromal cells (MSCs) have been under the spotlight of stem cell therapy because of its multi-lineage differentiation capacity (reviewed by Ankrum, et al., 2010), immunosuppressive effect (Nauta, et al., 2007), and increasingly importantly, its ability to secret trophic factors that induce tissue regeneration (reviewed by Ankrum, et al., 2010). According to the US Public Clinical Trials Database (U. S. National Institutes of Health, 2012), there is nearly 300 clinical trials exploiting MSCs for their therapeutic values. Most of the current clinical trials target diabetics, ischemia, myocardial infarction, inflammation, and immune diseases. The trial outcomes, on the other hand, are encouraging but not yet satisfactory. Implanted or infused MSCs often have low efficacy *in vivo*. It is reasoned that the improvement of MSC therapy is hindered by the limited understanding of MSC cell fate *in vivo* (reviewed by Ankrum, et al., 2010). The consensus on MSC identification is solely based on its marker expression and differentiation potential under *in vitro* conditions (Augello, et al., 2010; Dominici, et al., 2006). Although MSC *in vitro* characteristics are intensively researched upon, their *in vivo* counterpart still remains to be found (reviewed by Corselli, et al., 2012).

A few discoveries in recent years provide hints on the *in vivo* niche of MSCs. The first piece of evidence comes from the successful isolation of MSC from a wide spectrum of tissues. Conventionally extracted from bone marrow, MSCs have now been isolated from virtually all postnatal connective tissues, such as the adipose tissue, dental pulp, and so on (reviewed by Bianco, et al., 2008; da Silva Meirelles, et

al., 2006). These studies suggest that the *in vivo* source of MSC must be widely distributed across different tissues and organs.

Following this line of thought, several research groups have come up with the hypothesis that the *in vivo* MSC reservoir is most likely to be associated with the blood vessels, which is present in all tissues in the body. More specifically, they propose that MSCs in situ are perivascular. To prove this theory, perivascular cells have been isolated and purified by flow cytometric cell sorting. The sorted cells were shown to display a MSC marker profile, and to demonstrate adipogenic (Crisan, et al., 2008; Corselli, et al., 2012; Zannettino, et al., 2008), osteogenic (Sacchetti, et al., 2007; Crisan, et al., 2008; Corselli, et al., 2012; Zannettino, et al., 2008), chondrogenic (Corselli, et al., 2012; Zannettino, et al., 2008), and even myogenic potentials (Crisan, et al., 2008; Dellavalle, et al., 2007). Therefore, perivascular cells are shown to be *bona fide* MSCs. Some even go so far as to pose the question that if all MSCs are pericytes (Caplan, 2008).

Under such circumstances, pericytes, one of the perivascular cells and are found around small blood vessels (Gaengel, et al., 2009), have attracted great research interest. Until recently, pericytes have been a cell type that is not well studied and understood. They have been shown to play an essential role in the maturation and stabilization of blood vessels (Armulik, et al., 2005). The recent evidences on their additional function as MSC-like progenitor cells (reviewed by Crisan, et al., in press) put them under new attention as candidates for cell therapy and regenerative medicine. These cells, not only multipotent but also have pro-angiogenesis properties, may become a promising alternative for MSC in stem cell therapy. Also, the study on

the relationship between pericytes and MSCs may shine light on the obscure *in vivo* identity of MSC.

However, the identification of pericytes is no easier problem. Different from MSCs, pericytes are traditionally identified not by their *in vitro* characteristics, but by their *in vivo* location. Pericytes are defined as cells located within the basement membrane of endothelial cells. This is until now the ultimate standard for pericyte identification, which is unfortunately impractical and sometimes impossible to verify for *in vitro* cultures. Besides the definition, pericyte identification is further complicated by its heterogeneity. Pericytes are widely distributed around virtually all small blood vessels in the body, and their maker expression depends on their tissue of origin as well as degree of maturation of the associated blood vessels (reviewed by Bergers, et al., 2005). To date, there is no marker or combination of markers that is available for identification of pericytes from all tissues reviewed by (Armulik, et al., 2011). A vigorous study that claims to have isolated pericytes by a set of markers would often verify the *in vivo* location of the cells in their tissue of origin.

Most of the recent studies on pericyte-MSC relationship concentrate on flow cytometric sorting isolated pericytes, and their *in vivo* or *in vitro* characterization for MSC-specific features (Péault, et al., 2007; Crisan, et al., 2008; Covas, et al., 2008; Castrechini, et al., 2010; Corselli, et al., 2012). Side by side comparison of MSCs and pericytes are rare. For example, few papers have been published on comparing MSCs and pericytes from the same bone marrow source (reviewed by Bouacida, et al., 2012). However, such comparative assays are essential for finding out the differences and similarities of the two cell populations.

This study thus proposes an unbiased comparison between a typical pericyte population (pericytes from human placenta isolated by CD146 expression, Promocell) and a typical MSC population (MSCs isolated from human bone marrow by plastic adherence, Lonza) for MSC as well as pericyte related characteristics. In this way, this study aims to generate novel insights on several elusive aspects of the MSC-pericyte relationship:

The first motivation of the study is to address the unanswered question: are MSCs really pericytes? Although pericytes have been shown to possess the major characteristics of MSC (Crisan, et al., 2008; Dellavalle, et al., 2007; Shi, et al., 2003; Zannettino, et al., 2008; Díaz-Flores, et al., 2009), the reverse question is rarely posed. Do MSCs possess the typical pericyte features? Pericytes have been shown to interact with endothelial cells through a number of pathways, and to play a specific role in angiogenesis and blood vessel maintenance (Bergers, 2008; Bergers, et al., 2005; Hirschi, et al., 1996). These functions are rarely associated with MSCs, and would need to be verified before being able to conclude if MSCs are truly pericytes. That is why this study chose to test both pericytes and MSCs not only for MSC related characteristics, but also pericyte and angiogenesis related features.

The second motivation of the study is to seek a way to identify pericyte *in vitro*. By screening both pericytes and MSCs for a spectrum of marker and functional assays, we expect to establish a set of *in vitro* assays that is sensitive enough to distinguish pericytes from other mesenchymal lineages, for example MSCs, if there is any differences between the two. Many who claim that they have identified pericytes rely on one or a few markers, while to this day there is no pericyte specific/ pan-pericyte

marker available (reviewed by Armulik, et al., 2011). It is to be verified if these "pericytes", isolated from various tissues using different sets of markers, refer indeed to the same population. The ultimate test still requires verifying the *in vivo* perivascular location of the cells. It would be of great interest to have a set of standardized assays that enables identification of pericytes *in vitro*. Such assays would also need to be able to identify functional pericytes, i.e. cells that maintains their pro-angiogenic properties and the ability to interact with endothelial cells. This would provide a platform to differentiate pericytes from other cell populations in vitro. Moreover, it would also allow for standardization of pericytes for research purposes as well as for clinical application.

Besides providing a tool for facilitating future research, a third motivation of the study is to obtain insights of the *in vivo* characteristics of pericytes and MSCs. Although the in vivo function and properties of MSCs and pericytes are beyond the scope of this study, some clues may be obtained from their *in vitro* characteristics and behaviors.

## 1.1 Mesenchymal Stem Cells (MSCs)

Before moving on to compare the different cell types, it is important to review the current definition and methods of identification and characterization for each of them.

The cell population that is called mesenchymal stem cells today was first described by Friedenstein (1968), who found a non-hematopoietic progenitor population in the bone marrow that is capable of forming single clones in culture (colony-forming units-fibroblastic or CFU-Fs) and is capable to undergo osteogenesis *in vitro*

(Friedenstein, et al., 1970).The term “mesenchymal stem cells”, or MSCs, are later made popular by Pittenger et al. (1999), who showed that these plastic adherent, colony-forming cells isolated from bone marrow were able to differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro* when induced by a cocktail of small molecules. They further suggested that this particular cell population may be the reservoir for adult connective tissue regeneration. Nowadays the sources of MSCs have been expanded beyond bone marrow. MSCs have been isolated from virtually all types of postnatal tissues, such as adipose tissue, dental pulp, and so on (reviewed by Bianco, et al., 2008; da Silva Meirelles, et al., 2006). The *in vivo* location of MSCs still remains to be confirmed, which is difficult due to the lack of a MSC-specific marker set (reviewed by Bianco, 2011).

One of the currently most accepted definition of MSCs is proposed by the International Society for Cellular Therapy (ISCT) (Dominici, et al., 2006), who suggested three minimal conditions for a cell population to be called MSCs. Firstly, the cells have to be plastic adherent, Secondly, they should be positive for surface antigens CD105, CD73, CD90, and at the same time be negative for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR. Lastly, they should be able to differentiate *in vitro* into three mesenchymal lineages, namely osteoblasts, adipocytes, and chondrocytes, under standard differentiation conditions.

Recent years have seen a shift of interest in the clinical application of MSCs. MSCs were initially regarded as the earliest progenitor cells in mesenchymal lineage (Caplan, 1994). The earlier studies focused on their ability to self-renew and to differentiate into multiple mesenchymal lineages, and tried to explore their

therapeutic potential for tissue repair or even for gene therapy (Bonab, et al., 2006). Mesenchymal stem cells from bone marrow have already been used for clinical applications (Gerson, 1999). It has been since observed that MSC implantation resulted somehow in reduced inflammation, fibrosis and apoptosis, even when there is a lack of effective MSC differentiation *in situ* (reviewed by Ankrum, et al., 2010; Bianco, 2011). Systematically infused auto- or allogeneic MSCs were able to home to damaged tissues and to establish a conductive microenvironment for tissue regeneration. It has thus been suggested that other factors than differentiation and proliferation must be contributing to the therapeutic effect of MSC in clinical trials. However, the actual mechanism of the effect of MSCs *in vivo* is still unclear. (Ankrum, et al., 2010; Caplan, 2007; Bianco, 2011)

Although numerous clinical trials are ongoing to exploit the therapeutic effect of MSCs, few have proved to be significantly effective. It has been suggested that the current bottleneck of MSC cell therapy is the lack of understanding of their *in vivo* cell fate (Ankrum, et al., 2010). The dilemma is that the definition and characterization of MSCs have depended exclusively on *in vitro* cultures, leaving the *in situ* identity and behavior of these cells elusive (Bianco, 2011).

Even the nomenclature of MSC is now being challenged. The use of "stem cells" is considered not vigorous. MSCs only have limited renewing ability *in vitro*. Furthermore, proliferation and differentiation in culture do not necessarily mean self-renewal and multi-potency *in vivo* (Bianco, 2011). The word "mesenchymal" is also often debated, since muscle and bone are derived from different progenitors during the early embryonic development (Bianco, 2011; Nombela-Arrieta, et al., 2011)

Therefore, the search of the *in vivo* counterpart of MSC is an important ongoing research topic both for elucidating on the identity of MSCs as well as for improving the clinical outcome of MSC-based therapy. Pericytes, with numerous features shared with MSCs, may promise to provide valuable clues on the subject.

## 1.2 Pericytes

The discovery of pericytes is attributed to the French scientist Charles Rouget in 1873. They carried thus the name "Rouget cells". The term "pericytes" was first coined by Zimmermann in 1923, referring to their close association with endothelial cells (Armulik, et al., 2011; Hirschi, et al., 1996). The definition of pericytes has since depended heavily on the *in vivo* location of the cells relative the endothelial cells. Pericytes are originally defined as extensively branched cells located in non-muscular microvessels, capillaries and postcapillary venules (Díaz-Flores, et al., 2009). The currently accepted and most vigorous definition of pericytes is cells that are located within the basement membrane of blood vessels, which come from the electron microscopy observation of pericytes *in situ* (reviewed by Sims, 1986).

In the vasculature system, pericyte is one of the two categories of mural cells that are found around blood vessels (Figure 1). In specific, pericytes are found around small blood vessels. They wrap the selves around the inner single-layer vessel lumen formed by endothelial cells (EC). Pericytes are in physical contact with EC and have intimate interactions with the EC-formed vessels (McDonald, 2008). The other type of mural cells, smooth muscle cells, is found around large blood vessels. They form multiple layers (tunica media) around the endothelial cells-formed vessels (tunica

intia). They are further enveloped by the tunica adventitia, which consists of fibroblasts and connective tissue (Corselli, et al., 2010).

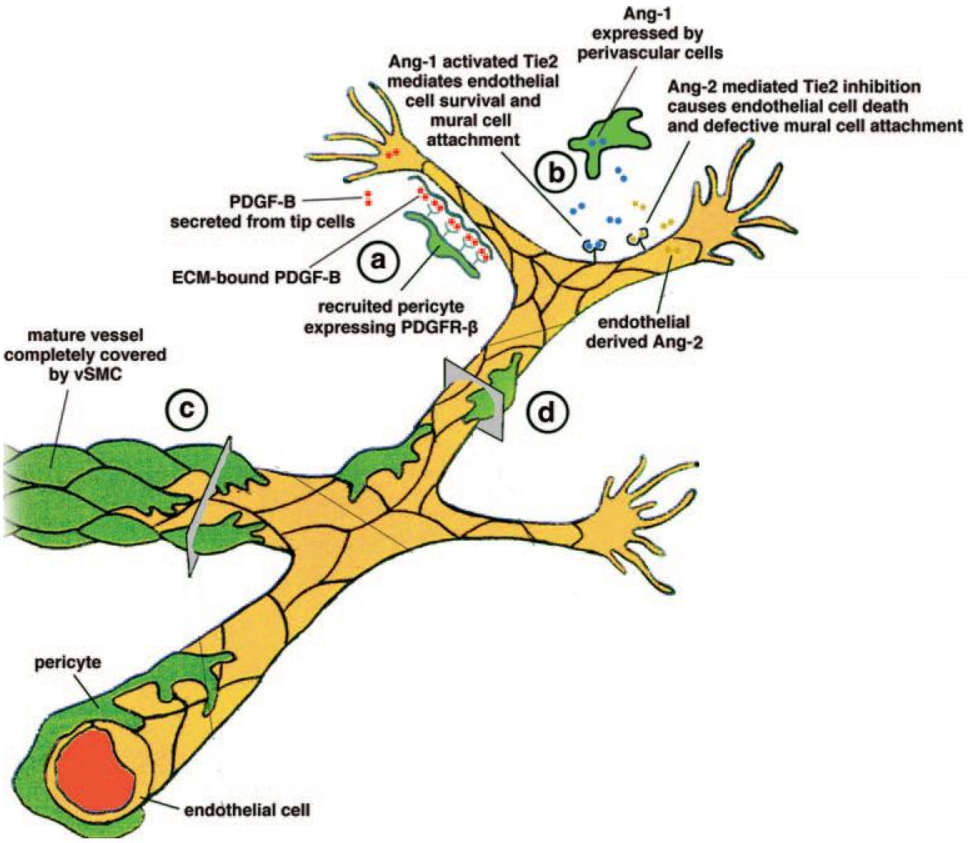



**Figure 1. EC-mural cell interaction** (adapted from (Gaengel, et al., 2009)). Blood vessels consist of two cell types: endothelial cells (EC, in yellow) which form the internal lumen, and mural cells (in green) which wrap around the EC-formed vessels. Under the class of mural cells, there is a sub-category of cells named pericytes (at lower left corner of the diagram) that are embedded within the basement membrane of blood vessels in close association with EC. The interaction and exchange of signal molecules between pericytes and EC are essential for the stabilization and maturation of small blood vessels. For example, the PDGF-B/PDGFR-β pathway and the Ang1/Tie2 pathway (represented by a and b, respectively).

The prominent feature of pericytes is that they sit in the basement membrane of the blood vessels. They are in close contact with the endothelial cell through various mechanisms such as gap junction or peg-socket contact (Armulik, et al., 2011; Hirschi, et al., 1996).

### 1.2.1 Pericyte distribution in tissues

Pericytes are widely distributed in the body. Pericytes are found in almost all tissue types in blood microvasculature, but not in normal lymphatic system (Armulik, et al., 2011). The most prominent feature is their close association with endothelial cell vessels. Pericytes are located more frequently around microvasculature such as capillaries and small venules, as well as pre-capillary arterioles (Sims, 1986). Pericytes are often found at the junction points of capillaries or of small vessels and capillaries, where they stretch themselves along the length of blood vessels across several branches (Armulik, et al., 2011; Bergers, 2008). The EC-pericyte ratio around blood vessels is tissue specific. It can vary from 1:1 in retina tissues and down to 100:1 in human skeletal muscle, for example (reviewed by Díaz-Flores, et al. (2009)). Besides the variation in the EC-pericyte ratio, pericyte distribution in tissue also varies in the form in which pericytes wrap themselves around EC. They can come in the form of single, discontinuous cells to a mono-cell layer around EC-formed vessels (Gerhardt, et al., 2003; Hirschi, et al., 1996)

Pericytes are found also at sprouting blood vessels. EC recruit pericytes during angiogenesis by secreting platelet-derived growth factor (PDGF), which promote the proliferation and migration of pericytes (Armulik, et al., 2005). Depletion of pericytes through inhibition of platelet-derived growth factor receptor β (PDGFR-β) in vivo leads to leaky and dilated vessels in mice as a results of lack of mural cells around the blood vessels (Hellström, et al., 2001).

So far, pericytes have been isolated from a wide spectrum of human tissues, such as skeletal muscle, myocardium, placenta, pancreas, skin, brain, and bone marrow (Crisan, et al., 2008), Zannettino and colleagues (2008) have isolated multipotent

pericyte-like cells from human adult adipose tissues by the markers STRO-1, CD146 or 3G5. However, it is worth noting that the isolated "pericytes" have a different marker profile compared to Crisan's group, and common pericyte markers, like desmin, NG2, PDGFR-β, has not been tested. The markers used for isolation are not restricted to small vessels, and the expression of STRO-1 was not exclusively perivascular, based on the immunofluorescence staining of frozen sections. Moreover, only a small portion of the isolated cells possessed multipotency. The group of Paolo Bianco (Dellavalle, et al., 2007) isolated ALP+ CD56- cells from human adult muscle that exhibited a typical pericyte marker profile (annexin V, alkaline phosphatase, desmin, smooth muscle actin, vimentin and PDGFR-β), though they have weak expression for CD90, CD105 and CD146. It demonstrates that pericytes isolated using different markers may have different marker profiles, while those isolated with CD146 resemble most that of MSCs.

### 1.2.2 Pericyte origin

Pericytes can develop from a variety of tissues (Lamagna, et al., 2006). For example, brain pericytes are shown to originate from neurocrest (Bergwerff, et al., 1998). It has also been proposed that VEGFR2+ angioblasts can differentiate into EC or pericytes under different stimuli (Yamashita, et al., 2000). There are also research groups who suggested that pericytes originate from myofibroblasts (D áz-Flores, et al., 2009). It has equally been shown that bone marrow derived cells, when systematically infused into mice, can home to perivascular locations, infiltrate with microvasculature, and express pericytic markers, indicating that some pericytes may also come from the bone marrow (Ozerdem, et al., 2005; Rajantie, et al., 2004)

Finally, MSCs have as well been proposed as pericyte precursors. It has been shown that when co-cultured with endothelial cells, MSCs (10T1/2, ATCC) are able to differentiate into pericyte-like phenotype. They expressed NG2 and αSMA, stabilized EC formed networks on matrigel, and homed to perivascular locations when implanted into mice developing vessels (Darland, et al., 2001; Hirschi, et al., 1998).

### 1.2.3 Increasing interest in pericyte research arising from newly discovered pericyte functions: an implication for their therapeutic potential

The research on pericyte function is still ongoing and recent years have seen rapid advances in understanding of the role of pericytes in microvascular system. Nevertheless, three main pericyte functions have been pointed out. The first function of pericyte is the maintenance of blood vessels through secreting growth factors that are indispensable for EC survival (Gaengel, et al., 2009; Gerhardt, et al., 2003). Three well-known ligand/receptor pairs in EC-pericyte interaction are VEGF/VEGFR, PDGF-B/PDGFR-βand Ang1/Tie2. Pericytes are able to produce vascular endothelial growth factor (VEGF) which binds to the VEGF receptors in EC. VEGF is essential for EC survival and regulates EC immigration (Darland, et al., 2003; Senger, et al., 1996; Franco, et al., 2011). PDGF-B is important for mural cell recruitment towards EC-formed vessels (Hellström, et al., 1999). Inhibition of PDGF-B impaired EC's ability to recruit mesenchymal cells to EC vessels on Matrigel™ *in vitro* (Hirschi, et al., 1998). Pericytes also secret Ang1, the main agonistic ligand for Tie2 receptor on EC (Gaengel, et al., 2009). Ang1/Tie2 pathway is shown to be essential for blood vessel maturation and stabilization. Mouse with Ang1 or Tie2 depletion died from cardiovascular failure as embryos (Suri, et al., 1996).

A second function of pericytes is to provide mechanical support and to control blood circulation through providing mechanical forces. Pericytes express a number of contractile proteins, for instance α-SMA, desmin and tropomyosin have been identified in pericytes *in vivo* or *in vitro* (Bergers, et al., 2005). Some research groups proposed that the pericytes are able to constrain blood vessels to contribute to the regulation of blood flow in small vessels (Rucker, et al., 2000; Bergers, et al., 2005). However, there is some controversy on if pericytes really act to provide contractile force to blood vessels, because there is a lack of direct evidence. Observation of pericyte contraction *in vivo* is a difficult issue, due to the lack of specific pericyte markers (reviewed by Armulik et al.) (2011).

Besides these two traditional functions, there is an increasingly popular theory that pericyte further processes the ability to serve as a reservoir of progenitor cells in different tissues (Augello, et al., 2010). As mentioned earlier, recent studies have reported that perivascular cells express MSC markers and possess multi-lineage differentiation potential (Crisan, et al., 2008; Covas, et al., 2008; da Silva Meirelles, et al., 2006; Shi, et al., 2003). As early as in 1988, it has been found that alkaline phosphates positive cells in the bone marrow are able to differentiate into adipocytes (Bianco, et al., 1988). More recently, pericytes derived from various tissues have been demonstrated to possess myogenic capacities (Crisan, et al., 2008). It has been further suggested that pericytes exhibit stem cell features and may even be mesenchymal stem cells (MSCs). It has been proposed that pericyte-like populations reside in a perivascular niche and may serve as local stem cell reservoirs (Crisan, et al., 2008; Zannettino, et al., 2008; da Silva Meirelles, et al., 2006; Shi, et al., 2003). It

is found that perivascular cells, isolated from adipose tissues by pericyte related markers STRO-1, CD146 or 3G5, expressed also stromal cell related markers (CD44, CD90, CD105, CD106, CD146, CD166, STRO-1, and alkaline phosphatase). These cells equally demonstrated the potential to differentiate into cells from different lineages (Zannettino, et al., 2008). This suggests that pericytes, besides their angiogenic properties, may also serve as a local stem cell source that response quickly to damaged tissues or growth signals in their proximity.

The group led by Bruno Péault in Pittsburgh published the ground-breaking article in Cell Stem Cell in July 2008 (Crisan, et al.), where they identified NG2, CD146, PDGFR-β as exclusive markers for cells at perivascular location. They thus isolated "pericytes" from different adult and fetal tissues by sorting for CD146+ CD34- CD45- CD56- population. They found that this cell population has the potential to differentiate into myogenic, osteogenic, adipogenic, and chondrogenic lineages, maintains the expression of pericytic markers NG2, CD146, and αSMA, as well as typical MSC antigens. They equally demonstrated by immunohistochemistry that MSC marker expressing cells were found in perivascular locations, and that they co-expressed CD146.

## 1.3 *in vitro* identification methods for MSCs

The international consensus for defining MSCs is by their three features: plastic adherence, marker expression, and multipotency (Dominici, et al., 2006).MSCs in culture are characterized by their plastic-adherent well-spread morphology (Pittenger, et al., 1999; Dominici, et al., 2006). Furthermore, there is a set of markers that are

generally agreed upon to be expressed by MSCs. MSCs are expected to express CD90 (Thy-1), CD105 (Endoglin), CD73, CD13 (APN) (Jiang, et al., 2002). At the same time, MSCs normally do not express CD11b (monocyte marker), CD45 (leukocyte marker), CD34 (hematopoietic stem cell marker), CD117 (c-kit, hematopoietic progenitor cell marker), CD19 (B cell marker), HLA-DR (antigen presenting cell marker), glycophorin-A, and CD31 (EC marker) (Kolf, et al., 2007; Dominici, et al., 2006).

### 1.3.1. Three MSC hallmark antigens CD90, CD105, and CD73

CD90, CD105, and CD73 are the three MSC markers that are part of the minimal criteria for defining MSC proposed by the International Society for Cellular Therapy (ISCT) (Dominici, et al., 2006). This publication has been intensively cited as a standard of MSC identification *in vitro*.

CD90, also named Thy-1, is an important surface glycoprotein that regulates cell-cell interactions (Rege, et al., 2006). MSCs are shown to express CD90 in culture (Pittenger, et al., 1999). It is expressed in fibroblasts, brain cells, thymocytes, T cells, myoblasts, epidermal cells and keratinocytes (Pont, 1987; Haeryfar, et al., 2004) . It is also found in activated endothelial cells, smooth muscle cells, and a restricted population of hematopoietic cells (Craig, et al., 1993; Haeryfar, et al., 2004). In fibroblasts, CD90 is found to affect cell proliferation, collagen production, and migration (reviewed by Rege, et al., 2006).

CD105 (endoglin), is a dimeric protein that form part of the transforming growth factor-beta receptor complex (Yamashita, et al., 1994). CD105 is strongly expressed

in vascular EC and plays a role in angiogenesis. It is also expressed in stromal cells and fibroblasts, as reviewed by Fonsatti (2001).

CD73 (ecto-5'-nucleotidase (S'-NT)) is an ecto-enzyme commonly found on the cell membrane which catalyzes the dephosphorylation of monophosphates (Resta, et al., 1998). It is found to be expressed in mesenchymal stem cells as well as in lymphocytes (Barry, et al., 2001).

### 1.3.2. MSCs frequently express CD29, CD13, CD166, and CD146

Integrins are the major surface adhesion receptors. They consist of αβ heterodimers (Hynes, 1992). CD29 is the integrin β1 subunit, which are the receptors for collagen (α1β1, α2β1, α10β1, α11β1), laminin (α3β1, α6β1, α7β1), and RGD (α5β1, αVβ1, α8β1), a tripeptide present in fibronectin and vitronectin (Hynes, 2002).Most of them are expressed in endothelial cells (Francis, et al., 2002). Integrins β1 are equally found in the center nervous system and are important for cerebral angiognenesis, especially α5β1 (Li, et al., 2012). All four integrin β1 isoforms are expressed in MSCs, with β1A showing the highest expression. (Ip, et al., 2007). As reviewed by (Francis, et al., 2002), β1 integrins or CD29 have been shown to play an essential part in vascular development. Angiogenesis is haulted after inhibition of α1β1 and α2β1 (Senger, et al., 1996).

CD13 is a membrane bound ectopeptidase named aminopeptidase N (APN) which contribute to the degradation of certain proteins and peptides. Besides its enzyme activity, it is also involved in other cell activities, especially in the migration, differentiation, and angiogenesis of malignant tumor cells (Wickström, et al., 2011).

The expression of CD13 is found in a wide range of cell types including epithelial, endothelial, and fibroblast-like cells. It is also strongly expressed in stem cells. It is used as a differentiation marker for granulocytes and monocytes, as reviewed by Bauvois, et al., (2006)

CD166, also named as activated leukocyte cell adhesion molecule (ALCAM), is a cell surface immunoglobulin. As its name suggests, CD166 is important for cell adhesion. It is expressed on hematopoietic progenitor cells, and endothelial cells, as reviewed by Ohneda, et al., (2001).

CD146 or S-endo 1 is a membrane glycoprotein that is located at the cell-cell contact point, and is possibly involved in cell-cell adhesion and cell-matrix interaction. CD146 is one of the markers that interest us the most, because it is often used for pericyte identification for research or commercial applications. It is reported to be expressed in EC, smooth muscle tissues, cerebellum, hair follicles of normal tissues, as well as melanomas and some other malignant tissues (Shih, et al., 1994). Recent discoveries have shown that CD146 is found in cells that co-express pericyte markers such as α-SMA and 3G5 (Shi, et al., 2003). Zimmerlin and colleagues and also shown that CD146+/CD31- cells identifies pericytes in tissue verified by histology (Zimmerlin, et al., 2009). CD146 has routinely been used as a marker for pericyte sorting from heterogeneous populations (Péault, et al., 2007; Crisan, et al., 2008; Covas, et al., 2008; PromoCell).

### 1.3.3. MSCs are supposed to be negative for EC markers CD144, and VEGFR2, and hematopoietic markers for CD45, CD34, and CD117

CD144, or VE-Cadherin, is the main adhesion molecule that is responsible for EC-EC cell junction. It is essential for the maintenance and regulation of cell-cell contacts and permeability of vessels. It is a specific EC marker (Vestweber, 2008).

Vascular endothelial growth factor receptors 2 (VEGFR2), or flk-1, is the major regulator of VEGF's mitogenic, angiogenic and permeability-regulation effect (Ferrara, et al., 2003). VEGFR2 is critical for the development of EC. It is mostly expressed in Vascular ECs and lymphatic ECs, while expression is also observable in neuronal cells, megakaryocytes and hematopoietic stem cells (Holmes, et al., 2007)

CD45 (leukocyte common antigen) is a common hematopoietic tyrosine phosphatase. It is the pan-leukocyte marker expressed in all hematopoietic cells but not mature erythrocytes. It is expressed in T cells and myeloid, and a subset of B cells (Nakano, et al., 2008). CD45 is involved in modulation of cell signaling and may control the immune cell response to external stimuli (Hermiston, et al., 2003).

CD34 is a surface protein commonly used to identify and isolate hematopoietic stem cells, (Nielsen, et al., 2008). None of the tested cell types expressed these two hematopoietic markers.

CD117, also named c-kit, is the stem cell factor (SCF) receptor. It is expressed in bone marrow derived hematopoietic stem cells, blood, mast cells, melanocytes, germ cells, neural cells, and human aortic endothelial cells, as (reviewed by Escribano, et al., 1998).

### 1.3.4. MSCs are not supposed to express histocompatibility antigen HLA-DR, monocyte related antigen CD11b, and B cell marker CD19

HLA-DR, the main histocompatibility complex (MHC) class II molecule, is essential for antigen presentation function in immune cells and is expressed in macrophages, dendritic cells, B-cells, monocytes, and progenitor cells (Oczenski, et al., 2003; Yoshiike, et al., 1991). HLA-DR is not expressed in resting T cells. However in some pathological conditions and in tissue culture T cells are found to be positive for HLA-DR, possibly due to activation (Yoshiike, et al., 1991).

CD11b (Mac-1) are leukocyte surface proteins and belong to the class $\beta_2$ of the integrin family (Mazzone, et al., 1995). It has been found in macrophages, monocytes (Springer, et al., 1979) as well as for granulocytes, natural killer cells, and a subset of T cells (McFarland, et al., 1992).

CD19 is the major component of signal transduction-complex with CD21, CD81 and CD225, and amplifies signals from B cell surface receptor. It is an exclusive marker for B cells found in bone marrow and in peripheral blood. (Tedder, 2009).

### 1.3.5. Functional assay for MSC characterization

MSC is characterized by its ability to proliferate and to differentiate *in vitro* into multiple mesenchymal lineages, such as osteoblasts, adipocytes, chondrocytes, among others. It is thus also necessary to show that the population is homogeneous rather than the combination of a few cell types, each committed to a different lineage (Pittenger, et al., 1999).

## 1.4. Pericytes identification in vitro

The vigorous definition of pericytes requires microscopic observation that the cells reside in the basement membrane of blood vessels. It has been recognized that "Pericytes" refer to different cell types that are found in the perivascular location. The location based definition often leads to confusion between pericytes and other perivascular mesenchymal cell populations such as SMC, fibroblasts, and MSCs. In practice it is also impossible to implement in *in vitro* conditions, and a compromised identification using morphology and the expression of a combination of markers is often used. Therefore, the characterization and identification of pericytes still remains a subject of research, as reviewed by Armulik, et al. (2011). Moreover, the difficulty to isolate a pure pericyte population makes it hard for studying the vascular formation process (Yamashita, et al., 2000).

### 1.4.1. Markers

Most of the pericyte markers are closely linked to the pericyte function. Some of the pericyte markers are molecules that are recognized to play an important role in EC-pericyte interactions, such as surface receptors VEGFR, Tie2, and PDGFR-β. Some contractile proteins, for example α-SMA, desmin, and tropomyosin, are also commonly used as pericyte markers *in vitro* and *in vivo*. There are also other surface antigens that are involved in vasculature, for example NG2 proteoglycan (Ozerdem, et al., 2001).

It is worth noting that up to date, there is no single marker that can be used to identify pericytes. The multiple marker profile, which is usually used instead for pericyte

identification, is neither exclusive nor stable, and depends on the tissue type as well as the stage of development of the cells (Armulik, et al., 2011; Díaz-Flores, et al., 2009; Lamagna, et al., 2006). Reviews containing lists of current and perspective pericyte markers can be found at (Armulik, et al., 2011; Díaz-Flores, et al., 2009)

α-SMA is one of the markers most frequently used for pericyte identification. However, it has been shown that α-SMA is a late pericyte marker which is expressed only in differentiated pericytes with smooth muscle cell phenotype. Therefore a low expression of α-SMA does not necessarily mean the lack of pericytes (McDonald, et al., 2003; Ozerdem, et al., 2003; Nehls, et al., 1991). α-SMA has also been shown to be expressed in MSC derived from murine tissues at variable levels (da Silva Meirelles, et al., 2006) as well as in fibroblasts (Hinz, et al., 2001).

PDGFR-β is the receptor of platelet-derived growth factor B (PDGF-B) that is released by EC during angiogenesis. It is expressed not only in pericytes, but also in stromal fibroblasts (Song, 2005). PDGFR-β plays en essential role in angiogenesis (Gaengel, et al., 2009; Rajkumar, et al., 2006). PDGFR-β or PDGFB knock-out in mice caused leaking vessels and can be lethal, with abnormal distribution of pericyte cells around the blood vessels. It is thus believed that PDGFR-β is essential for pericyte function of maintaining and stabilizing the vessels (Song, 2005; Levéen, et al., 1994; Soriano, 1994).

NG2 (neuron-glial antigen 2), sometimes called HMW-MAA (high molecular weight-melanoma associated antigen) in human, is a main transmembrane chondroitin sulfate proteoglycan. Ozerdem and colleague (Ozerdem, et al., 2001) have shown that NG2

is an exclusive pericyte marker in newly formed mouse microvasculature in vivo, and is expressed in α-SMA negative pericyte cells as well. It has also been shown that NG2 is expressed in cultured pericytes (Schlingemann, et al., 1990) as well as fibroblasts (Morgensterna, et al., 2003). Although the exact role NG2 plays in angiogenesis is not yet clear, it is known that it has strong affinity for basic fibroblast growth factor (bFGF) and PDGF-AA, and may thus be involved in cellular interaction (Ozerdem, et al., 2004).

Desmin, another contractile protein, is a myogenic marker that is expressed in all muscle cells (Li, et al., 1991). Together with α-SMA and NG2, desmin has been cited as "late" or mature pericyte markers (Song, 2005). Unlike α-SMA, desmin has been found to be expressed by pericytes both in the developing state and in its mature state (Verhoeven, et al., 1988; Nehls, et al., 1992). Nehls and colleagues have proposed that Desmin+ and α-SMA- cells represent developing pericytes (Nehls, et al., 1992). It has also been proposed that Desmin+/α-SMA- cells are pericytes and Desmin-/α-SMA+ cells are smooth muscle cells around the capillaries, and the expression of the two markers exclusive. Kurz and colleagues showed that both markers can be expressed by pericyte cells, although the α-SMA expression may appear weak in capillary pericytes (Kurz, et al., 2008).

Tie2 is the receptor for Angiopoietin-1 (Ang1). Experiments showed that mice depleted of TIE2 died from ruptured vasculature, highlighting the critical function of Tie2 (Puri, et al., 1995). Tie2 has been reported to be expressed by developing endothelial cells (Dumont, et al., 1992) and hematopoietic cells (Puri, et al., 2003), while its ligand Ang1 is expressed both on EC and pericytes (Wakui, et al., 2006).

Recent research suggested that retinal pericytes also express Tie2 receptor which may contribute to the control of pericyte survival (Cai, et al., 2008).

### 1.4.2. Functional assay

The fundamental characteristic of pericytes is its ability to interact with endothelial cells and contribute to the microvasculature remodeling process. Co-culturing assay, where pericytes and EC are cultured together in conditions that mimic the in vivo scenario, provide an approximation for studying pericyte behavior. In vitro functional models permit the control of different factors and to study their effect in angiogenesis, for instance, growth factors, signal molecules, cell type involved, and cell-to-cell ratios.

Different models exist for mimicking the *in vivo* process of angiogenesis. The simplest model is co-culture of two or more cell types in un-coated culture plates (Orlidge, et al., 1987). In 1980, EC were found to form spontaneously tube-like structure on collagen gel which resembles the *in vivo* EC behavior (Folkman, et al., 1980). The common model is to culture EC on plates coated with matrix protein such as Matrigel$^{TM}$ (2 D/3D model) or collagen (3 D) model (Bishop, et al., 1999; Koh, et al., 2008). Other in vitro models include organ-based models, for example the rat aortic ring assay, where a section of the rat aorta is cultured and the outgrowth of microvasculature can be measured (Ucuzian, et al., 2007). Another interesting assay involves the co-culture of ECs with smooth muscle cells (SMCs) in the form of cell spheroids in collagen gels (Korff, et al., 2001). The spheroids consisted of a mixture of ECs and SMCs. The sprouting of co-culture spheroids can thus be quantified by measuring the accumulative sprout length from each spheroid.

One of the most commonly used models is Matrigel™ angiogenic assay. Matrigel™ Basement Membrane Matrix is developed by BD Biosciences. The Matrigel™ is a decellularized, sterile, gel-like substance manufactured from the protein-rich matrix of a mouse sarcoma. Its composition is complex and contains different biological factors that may take part in the angiogenic assay. It contains matrix proteins including laminin and collagen, as well as a mixture of growth factors such as TGF-β, EGF, and FGF that are produced by the sarcoma cells. It is recommended for use in cell differentiation as well as in *in vitro* and *in vivo* angiogenic assays (BD, 2008) . Matrigel™ is equally used for in vivo plug assay where it is incorporated with biological factors and injected into animal models. This is one of the most common in vivo angiogenic assay (Kleinman, et al., 2005).

Matrigel™ possess a few valuable properties which makes it a suitable model for angiogenesis. The most important is its ability to induce EC to form inter-connecting tubular networks with a lumen, whose morphology resembles very much the in vivo capillary structure (Figure 2). The networks are usually formed within 6 to 12 hours and serve as a rapid test for the pro-angiogenic or inhibitory effect of drugs and biological factors (Kleinman, et al., 2005). The relatively rapid experimental process (within 24 hours) allows for a preliminary *in vitro* study of the angiogenesis process. Furthermore the 2D surface allows for convenience photo taking and quantification of results.

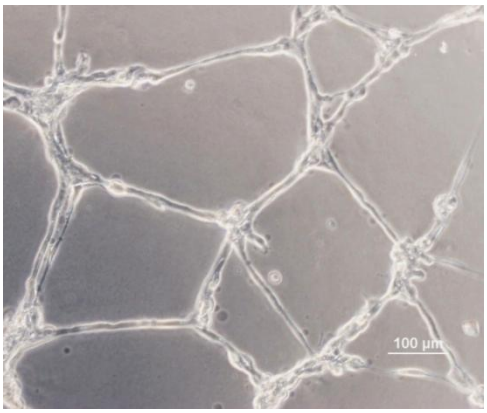



**Figure 2. EC form capillary-like networks when cultured on Matrigel™.** EC were seeded on Matrigel™ at 30,000 cells/cm$^2$. Phase contrast photo taken at 12 hours after seeding. Scale bar represents 100 µm

To date, EC has either been cultured alone on Matrigel™ to test the effect of pharmaceutical or biological molecules, or in co-culture with fibroblast to examine their interaction (Donovan, et al., 2001). Song and colleagues co-cultured endothelial cells with PDGFR-β positive perivascular cells on Matrigel™ and showed the co-localization of the two cell types at 18 hours and 3 days time points. The experiment showed the co-localization of pericytes with EC-formed capillaries *in vitro*. However they did not verify if this is a pericyte-specific behavior by comparing the isolated PDGFR-β expressing cells to other mesenchymal cell types (Song, 2005). Darland and D'Amore (2001) have equally used a co-culture of EC with MSC on Matrigel™, and have noted the formation of cord networks followed by aggregates formation at 24 hours. They therefore concluded that MSC adopted a pericyte-like phenotype in co-culture with EC.

## *2. Hypothesis and Objective*

The current methods for MSC and pericytes isolation and characterization are not able to answer the important question: although the isolated "pericytes" were tested for MSC and pericyte markers, as well as their multipotency, do these pericytes and MSCs maintain the pericyte related function, i.e., a role in angiogenesis and capillary maintenance? Also, there is a lack of studies that compare directly isolated pericytes and MSCs.

We therefore hypothesize that differences between pericytes and MSCs exist, and they may be related to the pro-angiogenic function of pericytes, which has been rarely tested for on MSCs.

**In a nutshell, we propose that pericytes are not only MSCs, but furthermore possess characteristics that MSCs cannot substitute for.**

To verify this hypothesis, we first tested Pl-Prc, MSCs, as well as fibroblast for the generally accepted MSC marker profile. A typical MSC profile should show positive expression for MSC-related markers (CD90, CD105, CD73, CD29, CD13, CD166, and CD146).At the same time, the cells should be negative for endothelial specific markers (CD144 and VEGFR2), hematopoietic markers (CD45, CD34 and CD117), as well as macrophages, monocyte and B cell related markers (HLA-DR, CD11b and CD19).

We believe that in order to identify whether pericytes are MSCs, it must be demonstrated that pericytes not only exhibit MSC marker characteristics, but is also

capable of differentiating into other mesenchymal lineages. We accessed the ability of all three cell types to differentiate *in vitro* into three mesenchymal lineages, namely osteoblasts, adipocytes, and chondrocytes .

To verify the second part of our hypothesis, which is that pericytes demonstrate characteristics that are not present in common MSCs, we examined both the marker and functional pericyte-related characteristics of the three cell types. Immunocytochemistry of the pericyte-related markers, α-SMA, PDGFR-β, NG2, Desmin, and Tie2 was performed for all three cell types. All of the markers except for and Tie2 have been routinely used for identifying pericytes *in vivo* or *in vitro*, while Tie2 is a receptor that plays important roles in angiogenesis and that we believe may demonstrate some differences between pericyte and non-pericyte cell types.

With respect to pericyte-specific function, we used the conventional Matrigel$^{TM}$ angiogenic assay, where pericytes have been observed to co-localize with EC-formed networks when seeded on the Matrigel$^{TM}$ surface. We further observed and compared the EC network morphology To find potential differences in the angiogenic properties of the cell type tested.

# *3. Methods:*

## 3.1. List of antibody

**Immunocytochemistry Antibodies**

| Marker | Clone | Cat No. | Format | Dilution | Source |
|---|---|---|---|---|---|
| **Primary Antibodies** | | | | | |
| **Mature Pericyte Markers** | | | | | |
| **PDGFR-β** | Y92 | AB32570 | Rabbit monoclonal | 1:100 | Abcam |
| **NG2** | | AB5320 | Rabbit polyclonal | 1:100 | Millipore |
| **α -SMA** | 1A4 | M0851 | Mouse monoclonal | 1:100 | Dako Cytomation |
| **Desmin** | DE-U-10 | AB6322 | Mouse monoclonal | 1:100 | Abcam |
| **Pericytes on Freshly Formed Vessels** | | | | | |
| **TIE-2** | C-20 | SC-324 | Rabbit polyclonal | 1:25 | Santa Cruz |
| **Secondary Antibodies** | | | | | |
| **488 goat anti mouse** | | S34253 | | 1:400 | Invitrogen |
| **594 goat anti rabbit** | | A11072 | | 1:400 | Invitrogen |

**Flow Cytometry Antibodies**

| Marker | Clone | Cat No. | Isotype | Volume /50µL | Source |
|---|---|---|---|---|---|
| **Control** | | | | | |
| **Iso FITC** | G155-178 | 555573 | FITC Mouse IgG2a κ | 10µL | BD Pharmigen |
| **Iso FITC** | MOPC-21 | 555748 | FITC Mouse IgG1 κ | 10µL | BD Pharmigen |
| **Iso PE** | G155-178 | 555574 | PE Mouse IgG2a κ | 10µL | BD Pharmigen |
| **Iso PE** | MOPC-21 | 555749 | PE Mouse IgG1 κ | 10µL | BD Pharmigen |
| **MSC and EC Markers** | | | | | |
| **CD90 (Thy-1)** | 5E10 | 555595 | FITC Mouse IgG1 κ | 2.5 µL | BD Pharmigen |
| **CD105 (Endoglin)** | SN6 | 12-1057 | PE Mouse IgG1 κ | 2.5 µL | e-Bioscience |
| **CD29(FN receptor)** | MAR4 | 555443 | PE Mouse IgG1 κ | 10 µL | BD Pharmigen |
| **CD146 (S-endo 1)** | P1H12 | 550315 | PE Mouse IgG1 κ | 10 µL | BD Pharmigen |
| **VEGFR-2 (Flk-1)** | 89106 | 560494 | PE Mouse IgG1 κ | 10 µL | BD Pharmigen |
| **CD144(VE-cadherin)** | 55-7H1 | 560411 | FITC Mouse IgG1 κ | 10 µL | BD Pharmigen |
| **Hematopoietic Markers** | | | | | |
| **CD45** | HI30 | 555482 | FITC Mouse IgG1 κ | 10 µL | BD Pharmingen |
| **CD13 (APN)** | WM15 | 560998 | PE Mouse IgG1, κ | 10 µL | BD Pharmingen |
| **CD34** | 581/CD34 | 555821 | FITC Mouse IgG1, κ | 10 µL | BD Pharmingen |
| **CD73 (5' –NT)** | AD2 | 550257 | PE Mouse IgG1, κ | 10 µL | BD Pharmingen |
| **CD166 (ALCAM)** | 3A6 | 559263 | PE Mouse IgG1, κ | 10 µL | BD Pharmingen |
| **CD117 (c-kit)** | 104D2 | 340529 | PE Mouse IgG1 κ | 10 µL | BD Bioscience |
| **Monocyte Markers** | | | | | |
| **CD11b** | D12 | 347557 | PE Mouse IgG2a, κ | 10 µL | BD Bioscience |
| **B/T Cell, Dendritic Cell markers** | | | | | |
| **CD19** | HIB19 | 555412 | FITC Mouse IgG1, κ | 10 µL | BD Pharmingen |
| **HLA-DR** | G46-6 | 556643 | FITC Mouse IgG2a, κ | 10 µL | BD Pharmingen |

**Table 1. List of antibodies used for immunocytochemistry and flow cytometry**

## 3.2. Cell Culture

Human placenta pericytes (Pl-Prc) (PromoCell, C-12980), human mesenchymal stem cells (MSCs) (Lonza, PT-2501), human lung fibroblasts (FB) (ATCC, CCL-186, strain IMR-90), and human umbilical vein endothelium cells (HUVEC) (Lonza, C2519A) were thawed and cultured until the desired passage, using their respective culture media, detachment kits, and standard protocols.

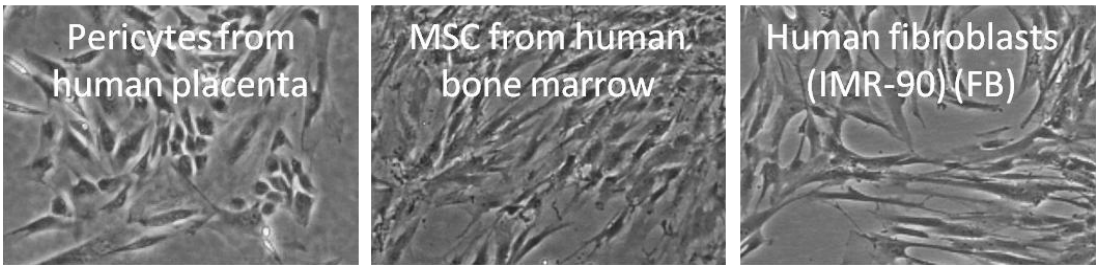



**Figure 3. Microscopic photos of cells in culture.**

| **Cell type** | **Media** | **Detachment kit** |
|---|---|---|
| Human placenta pericytes (Pl-Prc) (PromoCell, C-12980) | Pericyte Growth Medium (PGM) (PromoCell, C-28040) | DetachKit (PromoCell, C-41200) |
| Human mesenchymal stem cells (MSCs) (Lonza, PT-2501) | Low Glucose DMEM (Gibco 10569) supplemented with 10% FBS and 1% antibiotic-penicillin/streptomycin (p/s) | TrypLE™ (Gibco 12604-021) |
| Human lung fibroblasts (FB) (ATCC, CCL-186, strain IMR-90) | High Glucose DMEM (Gibco 10567) supplemented with 10% FBS and 1% p/s | TrypLE™ (Gibco 12604-021) |
| Human umbilical vein endothelial cells (HUVEC) (Lonza, C2519A) | CC-3156 EBM-2 Endothelial Basal Medium-2 (Lonza) supplemented with CC-4176 EGM-2 SingleQuots (Lonza) | Trysin/EDTA (Lonza, Cat. No. CC-5012)<br>Trypsin Neutralizing solution (TNS) (Lonza, Cat. No. CC-5002) |

**Table 2. Cell types used and their respective media and detachment kit**

Pericytes (promocell) are isolated from microvessels of the human placenta. More specifically, they are isolated from the chorionic villi of theplacenta tissue. They are CD146+, CD105+, CD31-, CD34-. The cells are sold and delivered at passage 2 (p2) in serum –free freezing medium. Cells were thawed, cultured, and passaged according to the product manual (Promocell).

MSC (Lonza) are isolated from human bone marrow. Cells were tested for their osteogenesis, chondrogenesis, and adipogenesis capacity. Cells were equally tested for their marker expression (CD105+, CD166+, CD29+, Cd44+, CD14-, CD34-, CD45-). The cells are sold and deliverd at passage 2 (p2). Cells were thawed, cultured and passaged according to the product manual (Lonza, 2011)

In this study, the cells after the first subculturing are refered to as p+1, which is equivalent to passage 3 (p3). Subsequently, cells after the second subculturing are refered to as p+2 (p4), and so on.

### 3.3. Flow Cytometry

Each cell type was cultured in three separate culture flasks to produce three independent sample sets. Cells were harvested at confluence and resuspended in an appropriate volume of flow cytometry buffer (1% FBS in PBS or HBSS). Each sample would require 60,000 to 200,000 cells in 50 µl flow cytometry buffer. 50 µl of the well mixed cell suspension was pipetted into a pre-labeled eppendorf tube for each sample. The respective antibody was mixed by flicking or vortexing before being added to each sample. The samples were incubated for 1 hour at 4 ℃ in dark, with gentle agitation. At the end of the incubation, 500 µl of the flow cytometry

buffer was added per sample and well mixed. The samples were centrifuged at 200g for 5 minutes (4 minutes for Pl-Prc), the supernatant discarded, and the pellets resuspended in 500 µl of ice-chilled 1% formaldehyde. The samples were filtered before being transferred into centrifuge tubes (BD 352058) and used for flow cytometry (Cyan ADP flow cytometer, Beckman Coulter).

For flow cytometry, the percentage of cells that showed positive staining compared to control is calculated. The exact gating (i.e. the fluoroscence treshold above which a cell is considered to be positively stained) is shown in Figure 3 to Figure 5. The percentage of positively stained cells is given by the number of cells with potive staining (compared to control) divided by total number of cells analyzed.

## 3.4. Differentiation

For osteogenesis, cells were plated in 24-well plates at 2,000 cells/well in their respective media, before switching on the following day to the inducing medium containing High Glucose DMEM (HG DMEM) containing 10% serum, 1% p/s, 100 nM Dexamethasone, 100 µM Ascorbic Acid, and 10 mM β-glycerophosphate. Inducing media were changed each 3 to 4 days. After 28 days, cells were fixed in 4% formaldehyde at room temperature, washed with PBS, and incubated with Alizarin Red for 10 min for staining of calcium deposits. Wells were then washed with deionized water and allowed to air dry inside the fume hood.

The seeding density (1,000 cells/ $cm^2$) is optimized from the protocol provided by the supplier (3,100 cells/$cm^2$) (Lonza, 2011; Salasznyk, et al., 2004; Schoolmeesters, et al., 2009). A low seeding density was chosen because it reduces peeling-off of control

sample from the plate, which happens frequently towards the end of the differentiation assay. Similar densities ($1 \times 10^3$ to $2.5 \times 10^3$ cells/cm$^2$) have been used for osteogenesis of embryonic stem cell derived MSCs (Barberi, et al., 2006). The reduction of seeding density does not seem to prevent osteogenesis, as accessed by Alizarin Red staining at the end of the 28$^{th}$ day (Figure 7).

For adipogenesis, cells were plated in 24-well plates at 50,000 cells/well in their respective media. Cells were allowed to adhere and to grow until confluency (usually within 24 hours), before switching on the next day to the induction medium containing High Glucose DMEM (HG DMEM), 10% serum, 1% p/s, 0.5 mM IBMX, 1 µM dexamethasone, 0.2 mM indomethacin and 10 µg/ml insulin. The cells were cultured for 4 days in the induction medium, followed by a 3-day culture in the maintenance medium containing HG DMEM, 10% serum and 1% p/s. The cycle was repeated for 28 days and cells were fixed in 4% formaldehyde at room temperature, washed with PBS. The fixed cells were then incubated with Nile Red and DAPI solution for 30 min for staining of lipid droplets, before being washed and stored in PBS.

For chondrogenesis, 5 x $10^5$ cells were centrifuged in 15 ml conical tubes to form pellets. The pellets were cultured in induction medium containing High Glucose DMEM (HG DMEM) with GlutaMax, 10% serum, 1% p/s, 0.1 µM dexamethasone, ITS + Premix (BD), 25 µg/ml ascorbic acid, 1x MEM Sodium Pyruvate (Gibco), 4mM Proline, 10 ng/ml TGF-β3. The medium was changed three times per week. After 28 days, the pellets were fixed in 4% formaldehyde, dehydrated using a series of ethanol and xylene washes, and embedded in paraffin. 5 µm sections were

produced using Microtome, The pellets were rehydrated by a series of xylene, ethanol and water washes, and then stained with Alcian Blue and Fast Red which stain for sulfated glycosaminoglycans and nuclei, respectively.

Ficoll was added to both the induction and the maintenance medium.

## 3.5. Immunocytochemistry (ICC)

Cells were cultured on 24-well plates until 90% confluence and fixed with methanol. Fixed cells were incubated for 1 hour in 3% (w/v) bovine serum albumin (BSA) solution in phosphate-buffered saline (PBS). The BSA was then replaced by primary antibody diluted in PBS and incubated for 90 minutes at room temperature with gengle shaking. Subsequently, the cells were washed three times with PBS, shaking for 5 minutes during each wash, before incubation in antibody cocktail containing DAPI and the respective secondary antibodies for 30 minutes. Cells were again washed three times with PBS before representative photos were taken using Olympus IX71 inverted microscope and Olympus CP70 microscope camera. For each cell type, a well was incubated only with the secondary antibody, which served as the control sample. The brightness and contrast of the photos taken for each antibody were adjusted such that the control sample did not show visible staining. This is to ensure that there is no false positive due to unspecific staining of the secondary antibody. The same brightness and contrast value is used for the same antibody across different cell types. The antibodies used are detailed in Table 1.

## 3.6. Matrigel$^{TM}$ Co-Localization Assay

Matrigel™ was thawn overnight at 4 ℃ and seeded in pre-chilled 48-well plates at 150 µl per well. The coated plates were allowed to settle overnight at 4 ℃, before being transferred to 37 ℃ for at least 1 hour to induce polymeration of Matrigel™. Cells were harvested, labeled with live cell tracking fluorescence (PKH67/PKH26, Sigma-Aldrich), protocol as detailed in section 3.7 below. Cells were then seeded in the Matrigel™ coated plates in 250 µl of EGM medium. HUVEC was seeded at 30,000 cells per well, and other cell types were seeded at different ratios with respect to HUVEC. The formation of capillary-like tube formation was imaged using Olympus IX71 inverted microscope and Olympus CP70 microscope camera at 4, 8, 12 and 24 hours. The cells were fixed with 4% formaldehyde for at least 1 hour at room temperature and then washed and stored in PBS.

## 3.7. Live Cell Labeling

Cells were harvested and resuspended in Hank's balanced saline solution (HBSS) (Gibco 14175) for cell counting. The needed number of cells were centrifuged down at 200g, 5 min and then resuspended in fluorescent diluent (Sigma PKH67/PKH26) at $10^5$ cells/ 100 µl. Prepare fluorescent cocktail with 0.5 µl of fluorescent cell linker dye (Sigma PKH67/PKH26) per 100 µl diluent and mix well with the cell suspension at 1:1 (v/v). The samples were incubated at room temperature for 4 min and then 2 ml of Heat-Inactivated FBS (HI FBS) was added per sample. The samples were transferred to clean test tubes and centrifuged at 200g, 5 min and supernatant discarded. The pellets were washed twice by resuspending in HI FBS and centrifuging, before being resuspended in required media for further experiments.

# 4. *Results*

**Summary of marker expression profile of Pl-Prc, MSC, and fibroblast**

| Markers | Alternative name | Pl-Prc | MSC | IMR-90 |
|---|---|---|---|---|
| **MSC-related markers (flow cytometry, value in %) *** | | | | |
| **CD90** | Thy-1 | 90.3 ±4.2 | 98.6 ±0.8 | 99.31 ±0.4 |
| **CD105** | Endoglin | 97.8 ±1.4 | 98.5 ±0.8 | 98.7 ±0.4 |
| **CD73** | ecto-5'-NT | 98.2 ±0.1 | 98.4 ±0.5 | 99.4 ±0.2 |
| **CD29** | Fibronectin receptor | 98.8 ±0.5 | 98.4 ±0.6 | 99.7 ±0. 1 |
| **CD13** | APN | 99.2 ±0.1 | 98.8 ±0.7 | 92.3 ±3.9 |
| **CD166** | ALCAM | 96.6 ±0.7 | 98.8 ±0.3 | 99.4 ±0.1 |
| **CD146** | S-endo | 86.4 ±0.7 | 94.1 ±2.9 | 94.8 ±3.6 |
| **Hematopoietic markers (flow cytometry, value in %) *** | | | | |
| **CD144** | VE-Cadherin | 0.5 ±0.4 | 4.1 ±1.6 | 0.0 ±0.0 |
| **VEGFR2** | Flk-1 | 1.8 ±1.6 | 2.5 ±3.7 | 0.0 ±0.0 |
| **CD45** | Leukocyte common antigen | 0.0 ±0.0 | 0.0 ±0.0 | 0.1 ±0.2 |
| **CD34** | | 0.3 ±0.5 | 1.6 ±1.3 | 0.7 ±1.2 |
| **CD117** | C-kit | 0.5 ±0.8 | 30.9 ±25.4 | 5.0 ±8.7 |
| **Macrophage, monocytes and B, T cell related markers (flow cytometry, value in %) *** | | | | |
| **HLA-DR** | MHC class II molecule | 0.0 ±0.0 | 0.0 ±0.0 | 0.5 ±0.9 |
| **CD11b** | Mac-1 | 0.0 ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 |
| **CD19** | | 0.1 ±0.1 | 0.7 ±0.7 | 0.1 ±0.1 |
| **Pericyte-related markers (ICC)** | | | | |
| **α-SMA** | α smooth muscle actin | + | +++ | + |
| **PDGFR-β** | PDGFR-B receptor β | +++ | ++ | + |
| **NG2** | Neuron-glial antigen 2 | ++ | - | - |
| **Desmin** | | ++ | - | - |
| **Tie2** | Ang1 receptor | + | - | - |

**Table 3. Expression profile of Pl-Prc, MSC, and fibroblast (Flow cytometry and Immunocytochemistry).**

*** For flow cytometry, the values indicated refer to the percentage of cells that express positively the given antigen, as compared to control.**

## 4.1. Pericytes displayed a typical MSC antigen expression profile

### 4.1.1. Pl-Prc, MSCs and fibroblasts expressed the common MSC markers CD90, CD105, CD73, CD29, CD13, CD166, and CD146

Pl-Prc, MSCs and fibroblasts were tested for common MSC markers CD90, CD105, CD73, and CD29 by flow cytometry. All of the three cell types were strongly positive for these MSC markers (Figure 4).

All three cell types in the experiment, Pl-Prc, MSC, and fibroblast, expressed strongly CD90, CD105 and CD73. The only exception is that Pl-Prc expressed slightly lower level of CD90. The levels of expression of these three MSC markers are similar in MSCs and the negative control fibroblasts.

Four more markers commonly cited to be expressed in MSCs, namely CD29, CD13, CD166, and CD146, were also tested for flow cytometry on all three cell types.

Interestingly in this study, a high percentage ofPl-Prc, MSC, and FB cells showed apositive expression for CD146. For Pl-Prc, which were selected for CD146 expression from placenta tissue, only about 86.4% of cells were positive for CD146. Additional testing was performed for Pl-Prc with an older passage (Figure 3a). The percentage of CD146 positive cell in pericyte population decreased drastically with increasing cell passage. Only 11.6% of cells of Pl-Prc p+5 ($5^{th}$ passage of the PromoCell p2 placenta pericytes. Or passage 7) showed positive expression for

CD146, compared to 86.4% for Pl-Prc p+3 (3rd passage of the stock cell, or passage 5).

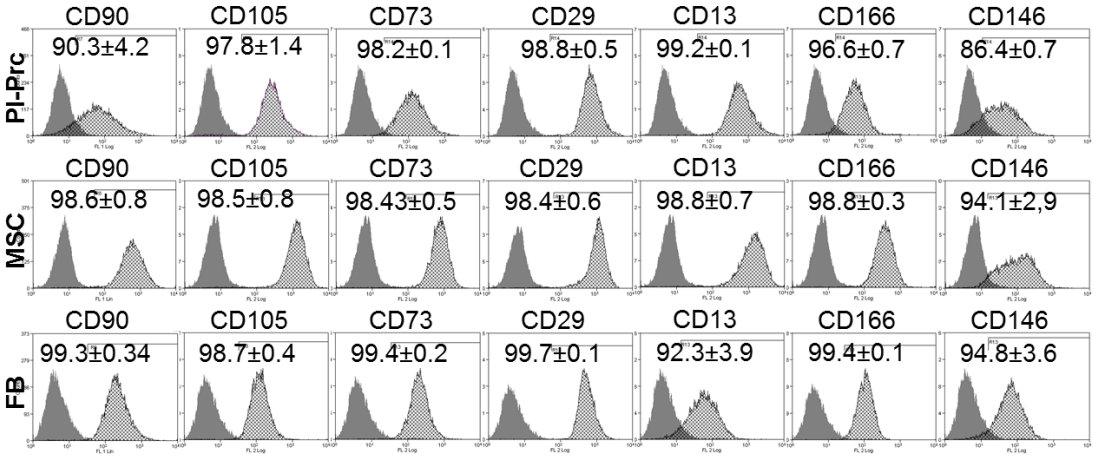



**Figure 4: Pl-Prc, MSC, and FB expressed MSC markers.** Results from flow cytometry (FC). Dark grey shade represents the conjugate control while light grey shade represents the sample marker expression. The percentage of cells that showed a positive staining compared to the conjugate control is shown for each FC antigen at the top of the diagram. Averages and standard deviations are calculated from three independent samples.

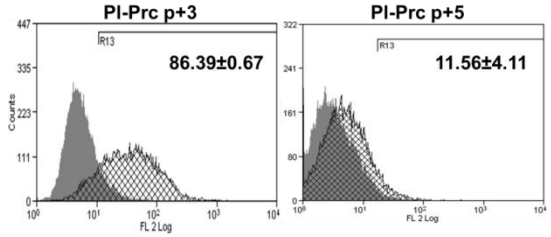



**Figure 4a. Decrease in CD146 expression in Pl-Prc with passage**. P+3 represents passage 5 and p+5 represents passage 7.

### 4.1.2. Pl-Prc, MSCs and Fibroblasts lacked the expression of endothelial markers and hematopoietic markers

Two of the previous markers expressed in MSCs, CD146 and CD105, are also commonly expressed in EC. Therefore in order to rule out the possibility of the contamination of EC in the cell culture, flow cytometry for two specific EC markers

were performed. Two hematopoietic markers were equally tested to make sure that no hematopoietic cells were included in the cell culture.

Pl-Prc, MSCs and fibroblasts did not express the endothelial cell markers CD144 and VEGFR2; neither did they express the hematopoietic markers CD45 and CD34.

CD117 was absent in Pl-Plc and fibroblasts from the flow cytometry results in this study. It was slightly positive for MSCs. The expression of CD117 is highly variable for MSCs, with a standard deviation of 25.38%..

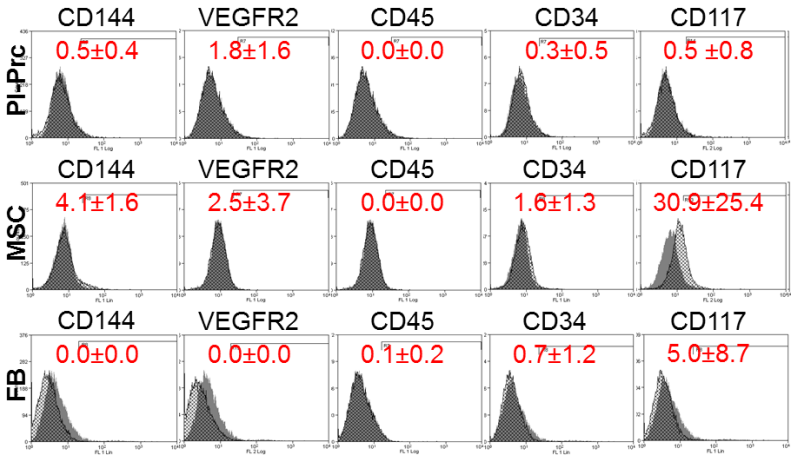



**Figure 5. Pl-Prc, MSCs, and FB lacked the expression of endothelial markers and hematopoietic markers.** Results from flow cytometry (FC). Dark grey shade represents the conjugate control while light grey shade represents the sample marker expression. The percentage of cells that showed a positive staining compared to the conjugate control is shown for each FC antigen at the top of the diagram. Averages and standard deviations are calculated from three independent samples.

### 4.1.3. Pl-Prc, MSCs, and Fibroblasts did not express macrophage, monocyte, and B cell related markers

As expected, none of the three cell types expressed the histocompatibility antigen HLA-DR and monocyte related antigen CD11b, and nor do they express the B cell

marker CD19. The percentages of cell population with positive expression for the three antigens are close to zero for all samples.

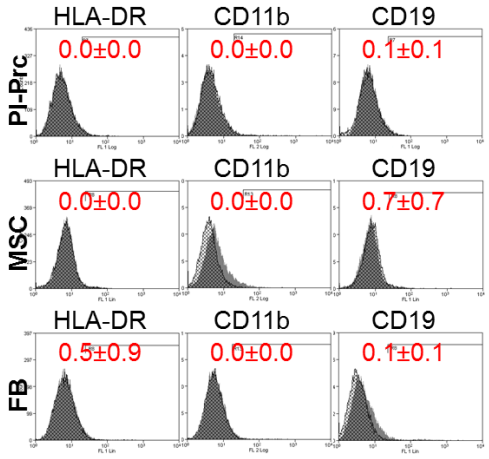



**Figure 6. None of Pl-Prc, MSCsand fibroblasts (FB) expressed the histocompatibility antigen HLA-DR, monocyte related marker CD11b, and the B cell markers CD11b and CD19** Results from flow cytometry (FC). Dark grey shade represents the conjugate control while light grey shade represents the sample marker expression. The percentage of cells that showed a positive staining compared to the conjugate control is shown for each FC antigen at the top of the diagram. Averages and standard deviations are calculated from three independent samples.

In summary, the antigen expression profile of the three cell type is very similar. It would be difficult to distinguish one cell type from another by looking at the MSC characteristic marker expression profile alone.

## 4.2. Pericytes demonstrated multipotent differentiation potential

Pl-Prc, MSCs and fibroblasts were induced for 4 weeks in osteogenesis, adipogenesis and chondrogenesis media, respectively. For visualization of the differentiation assay results, Alizarin Red was used to stain the osteogenesis plates for calcium deposition, Nile Red used to stain the adipogenesis plates for lipid droplets, and Alcian Blue used to visualize the production of glycosaminoglycan for chondrogenesis.

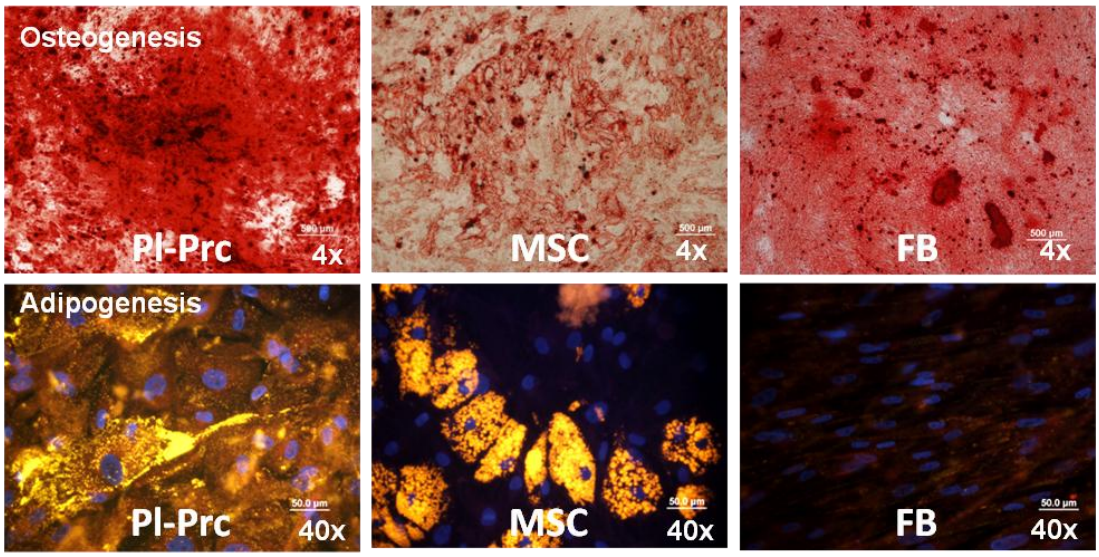



**Figure 7. Osteoblast and adipocyte induction of Pl-Prc, MSCs and fibroblasts (FB).** Upper row: osteogenic differentiation was visualized with Alizarin Red which stained for calcium deposit. Scale bar represents 500 μm. Lower row: adipogenesis was visualized with Nile Red, which stained lipid droplets. Nuclei were stained with DAPI. Scale bar represents 50 μm.

As shown in Fig. 7, all three cell types showed some level of calcification. The most $Ca^{2+}$ deposit is found in Pl-Prc, followed by fibroblasts and then MSCs. Pl-Prc and MSCs show similar pattern of the $Ca^{2+}$ deposit, with nodules of staining in the centre

and less intensive staining at the sides. Interestingly, fibroblasts were also able to produce mineral deposit under induction conditions. However, induced fibroblasts showed a different morphology, as shown by Alizarin Red staining (Figure 7). Instead of the typical mineral nodules as for Pl-Prc and MSCs, fibroblasts showed a more uniform $Ca^{2+}$ deposit pattern with occasional patches of dark red staining, which is different from the patterns of proper osteogenesis. This would be discussed in more details in the discussion session.

For adipogenesis, both Pl-Prc and MSCs produced rounded-up cells with lipid droplets. Fibroblasts, on the other hand, did not show any sign of adipogenesis. Adipogenesis in Pl-Prc shows a slightly different morphology from MSCs, with smaller lipid droplets and less regular cell shape, while MSCs produced large, round lipid droplets. However, most of the cells did produce lipid droplets, as opposed to MSCs where only a subpopulation showed adipogenic differentiation.

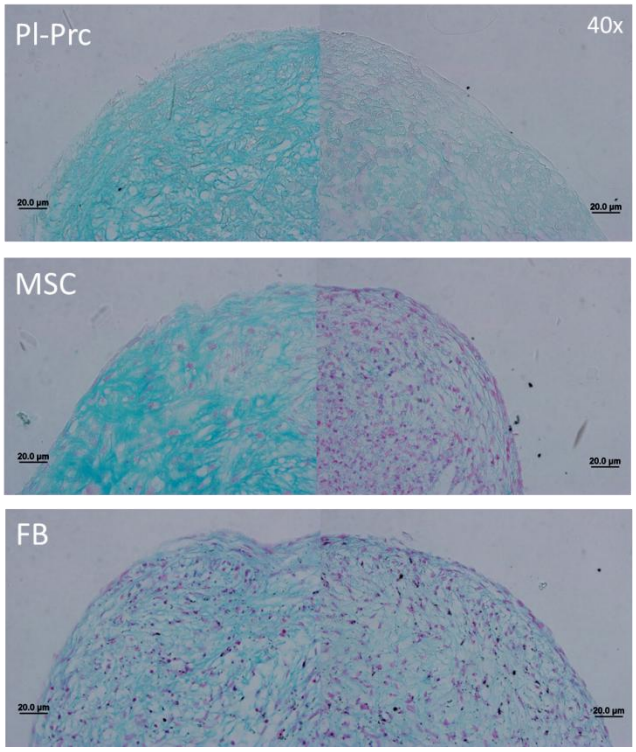



**Figure 8. chondrocyte induction of Pl-Prc, MSC, and fibroblast.** Chondrogenic differentiation was visualized with Alcian Blue which stained for glycosaminoglycan (GAG) production. Scale bar represents 20.0 µm. The left panels show the induced sample while the right panels show the control samples.

For chondrogenesis, Pl-Prc, MSCs and fibroblasts were cultured as pellets in induction media, over a period of 28 days. The induction protocol was optimized from current literature (Corselli, et al., 2012; Farrington-Rock, et al., 2004). Alcian blue was used for visualizing glycosaminoglycan (GAG), which is an indicator of chondrogenesis. Both MSC and Pl-Prc showed a positive Alcian Blue staining (Fig. 8) compared to control (right panels), while fibroblast showed little difference between the induced (left panel) and the control (right panel) sample.

Therefore, Pl-Prc do demonstrate the main MSC characteristics *in vitro* , which includes the expression of a specific marker panel, as well as the ability to differentiate into different lineages in vitro (Dominici, et al., 2006). So far, by MSC-related marker panel and the differentiation assay, there is no clear difference between Pl-Prc and MSCs. It is interesting to note that although fibroblasts are indistinguishable from Pl-Prc and MSC by the MSC-related marker panel, they fail to pass the functional assay, which is the *in vitro* differentiation into different lineages.

## 4.3. Pl-Prc expressed pericyte-related markers that MSCs lacked

Pl-Prc, MSCs and fibroblasts had a positive staining for α-SMA, which was strongest in MSCs. MSCs showed a staining pattern of parallel aligned intracellular fibers in all cells, whereas only a proportion of Pl-Prc and fibroblasts showed a staining, which was either granular or showed a less pronounced pattern of intracellular fibers.

PDGFR-β was expressed by all three cell types in this experiment, with the weakest staining in fibroblasts. Interestingly, the distribution of PDGFR-β staining was different in pericytes, showing a granular pattern around the nucleus approximately at endoplasmic reticulum location. MSC and fibroblast had a staining distributed over the whole cell body with stronger staining at cell-cell borders. Despite that, Pl-Prc's level of expression of PDGFR-β is the highest among the three cell types

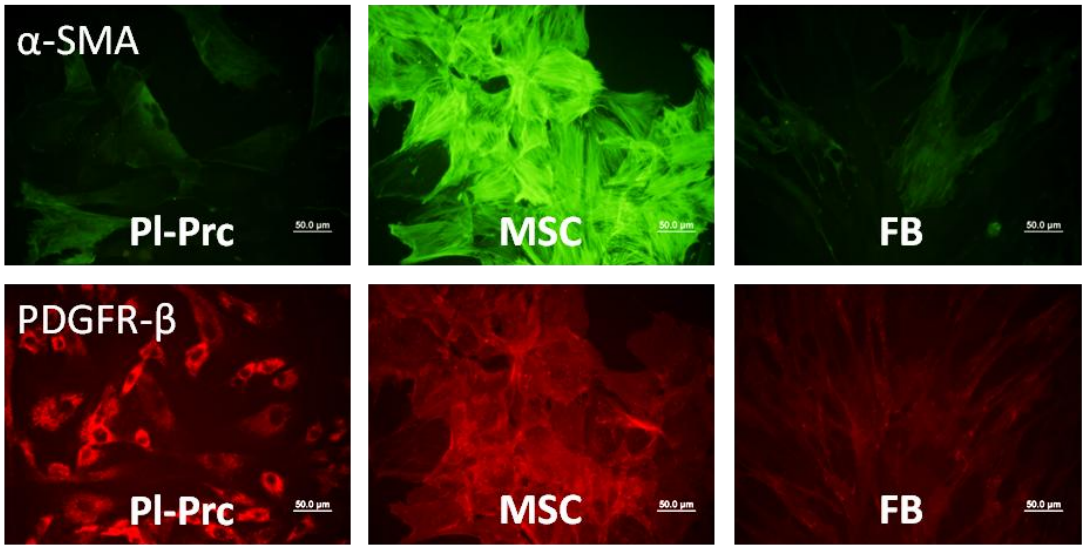
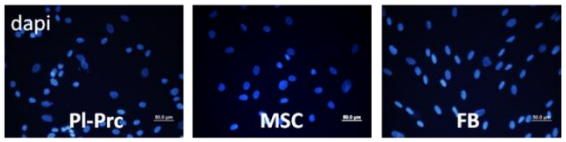



**Figure 9: Pl-Prc, MSCs, and fibroblasts all expressed pericytic markers α-SMA and PDGFR-β.** ICC results for α-SMA (green) and PDGFR-β (red) for Pl-Prc, MSC, and fibroblast (FB) respectively. Scare bar represents 50 µm. The corresponding Dapi nuclear staining is shown below.




NG2 expression is present in Pl-Prc, but not in MSCs and fibroblasts. Similar to PDGFR-β staining in Pl-Prc, NG2 was found principally around the nucleus approximately at endoplasmic reticulum location.

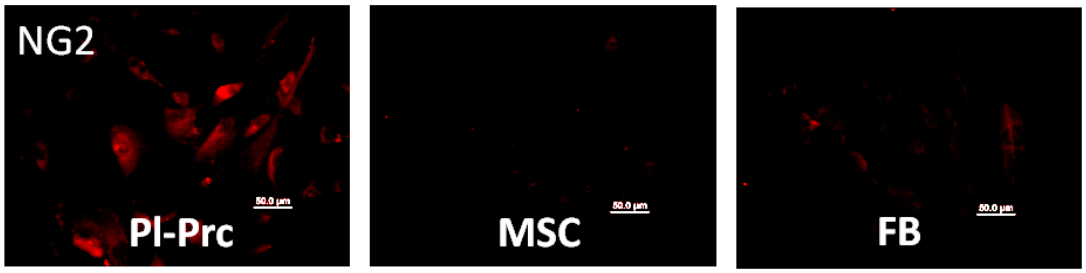
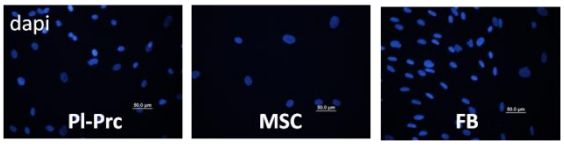
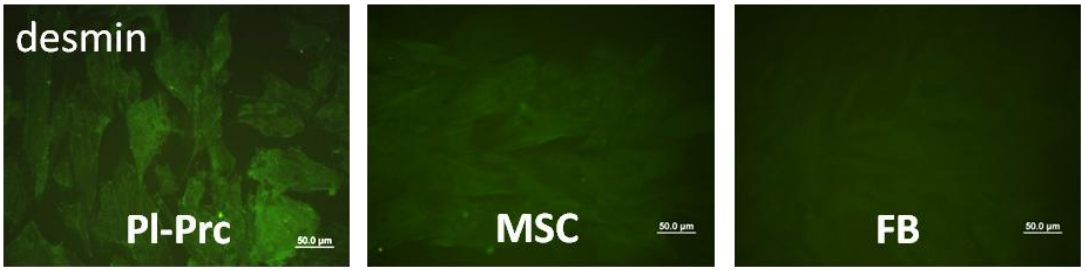



**Figure 10. NG2 expression is weak in all three cell types.** ICC results for NG2 (red) for Pl-Prc, MSC, and fibroblast (FB) respectively. Scare bar represents 50 µm. The corresponding Dapi nuclear staining is shown below.




Pl-Prc exhibited the strongest desmin expression among the three cell types. MSC showed a very weak staining, while fibroblasts were clearly negative for desmin

expression. Desmin in Pl-Prc was found at the cell surface. Fiber-like staining can be visualized, which indicate the presence of functional desmin fibers.



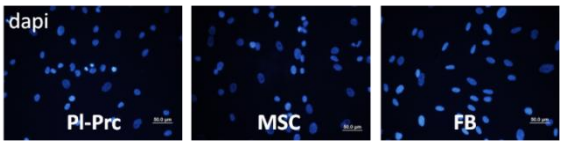




**Figure 11: Pl-Prc showed the strongest expression of desmin.** ICC results for desmin (green) for Pl-Prc, MSC, and fibroblast (FB) respectively. Scare bar represents 50 µm. The corresponding Dapi nuclear staining is shown below.

Tie2 is only expressed in Pl-Prc among the three cell types. From Figure 12, it can be seen that the staining for Tie2 in Pl-Prc was found mostly around the nuclei, likely to be at the Golgi apparatus. There was no observable staining for MSCs and fibroblasts, even though the cell densities were similar for all the three cell types, as shown by the DAPI nuclear staining.

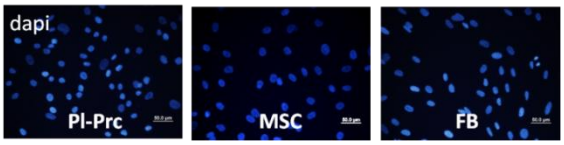




**Figure 12. Pl-Prc showed positive staining for TIE2**, ICC results for Tie2 (red) for Pl-Prc, MSC, and fibroblast (FB) respectively. Scare bar represents 50 µm. The corresponding Dapi nuclear staining is shown below.

## 4.4. Only pericytes maintained EC-formed network in Matrigel™ angiogenic assay

### 4.4.1. Only EC was able to develop networks on Matrigel™ alone.

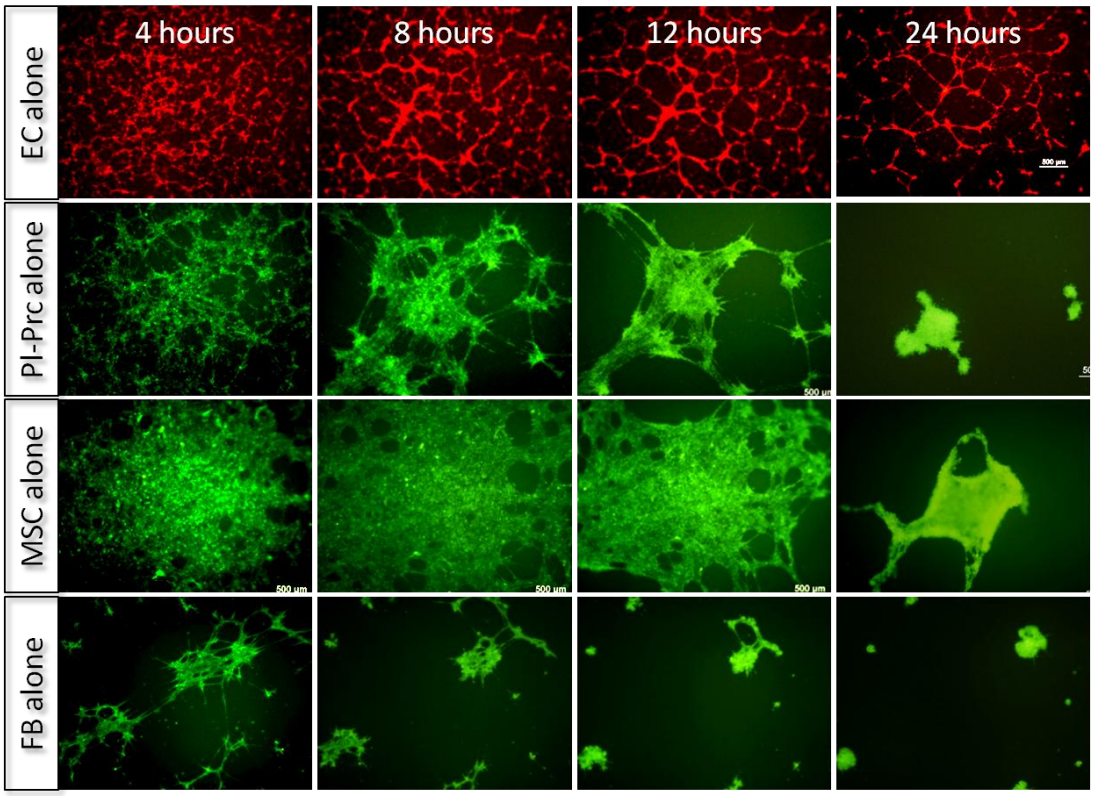



**Figure 13. Only EC formed networks when cultured alone on Matrigel™.** EC, Pl-Prc, MSCs, and fibroblasts were seeded separately at 30,000 cells per $cm^2$ per well on Matrigel™. Photos were taken at 4 hours, 8 hours, 12 hours, and 24 hours after seeding, respectively. Cells were labeled with PKH26 or PKH67 (refer to Materials and methods 3.7 Live cell labeling). Row 1: EC monoculture (red). Row 2-4: Pl-Prc, MSCs, and fibroblasts monoculture, respectively (green).

First of all, a mono-culture of the different cell types was performed on Matrigel™. Cells were seeded separately on the surface of a thin layer of Matrigel™ coating. The seeding density is 30,000 cells per $cm^2$. Among EC, Pl-Prc, MSCs and fibroblasts, only EC were able to form capillary-like networks, which remained stable for up to 24 hours.. The other cell types formed cell layers, which quickly arranged into smaller cell aggregates. They were interconnected by single cells, and finally contracted into bigger cell aggregates. All happened within 24 hours, however the time frame was cell specific. Fibroblasts show the fastest rate of network evolution,

where almost no network structure managed to be formed. They are followed by that of Pl-Prc, and then MSCs.

### 4.4.2. Pl-Prc, MSCs, and fibroblasts co-localized with EC-formed network

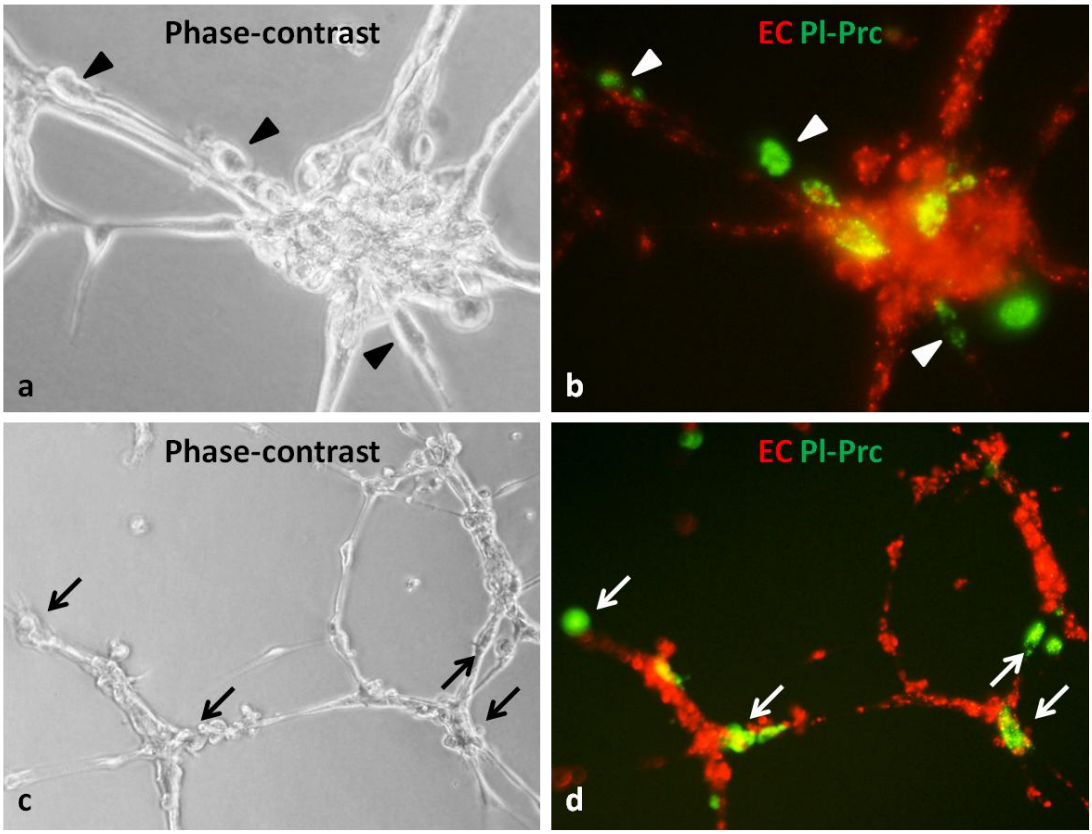



**Figure 14. Pericyte co-localize with EC-formed networks on Matrigel™ *in vitro*.** Left column (a &c) shows phase contrast photos and right column (b &d) shows cells labeled with fluorescent cell linker dye (as described in figure 13), where EC were labeled with red fluorescent dye and Pl-Prc green. (a) & (b) Pl-Prc co-localized with EC-formed tubular structure and attached themselves along the tubes. (c) & (d) Pl-Prc co-localized with the junction points of the EC-formed network. Photo taken at 12 hours after seeding on Matrigel™.

When EC and Pl-Prc are co-cultured on Matrigel™, the Pl-Prc co-localized with EC-formed networks and resided preferably at the junction points or along the length of the EC-formed vessels (Figure 14) This phenomenon is similar to what Song and colleague (2005) described in their work.

To compare the ability of Pl-Prc, MSCs, and fibroblasts to co-localize with EC-formed vessels on Matrigel™, experiments were repeated with MSC and fibroblasts (Figure 15).

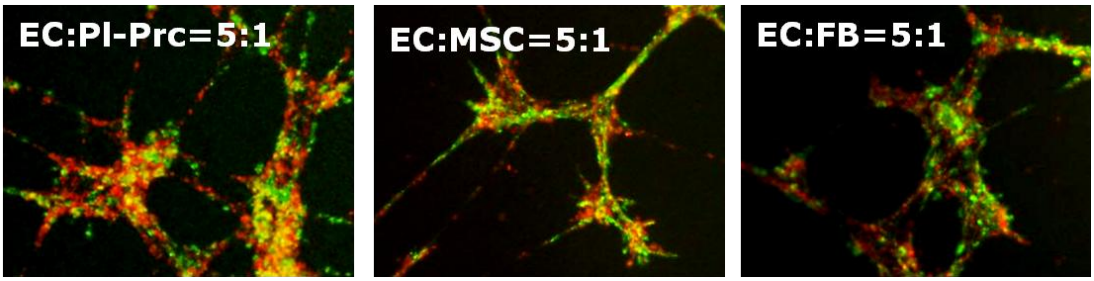



**Figure 15. Pl-Prc, MSCs, and fibroblasts (green) all co-localized with EC (red) formed network on Matrigel™.** EC were seeded in co-culture with Pl-Prc, MSCs, and fibroblasts, respectively. EC were seeded separately at 30,000 cells per $cm^2$ per well on Matrigel™. Photos were taken at 12 hours after seeding. Cells were labeled with fluorescent cell linker dye (as described in figure 13), where EC were labeled with red fluorescent dye and Pl-Prc, MSCs, and fibroblasts green.

Surprisingly all the three cell types (Pl-Prc, MSCs and fibroblasts) are able to co-localize with EC-formed networks. Their distributions with respect to EC are highly similar. They bound closely to EC-formed networks, and are either incorporated into or in co-localization with EC-formed tubes. They were found at the junction points as well as along the tubular structures. By observing the co-culture at the 12 hours time point, it is difficult to distinguishi between Pl-Prc, MSCs, and fibroblasts.

### 4.4.3. Pl-Prc maintained the EC networks over time

To further analyze the behavior of the different mesenchymal cell types tested, the co-culture assay was repeated with different cell ratios and time points (Figure 16 to Figure 19). In contrast to the 12 hours time point co-culture experiment (Figure 15), here the differences between Pl-Prc and the other two cell types were much more pronounced. At high cell densities (EC: Pl-Prc/MSC/FB = 2:1), only Pl-Prc were able to maintain tubular networks even at 24 hours after cell seeding although less and

thicker tubes remained, whereas for MSCs and fibroblasts, the networks were totally contracted away at 24 hours. For lower cell densities (EC: Pl-Prc/MSC/FB = 20:1), the EC-Pl-Prc co-culture networks resembles the networks formed by EC alone (Figure 13). While for MSCs and fibroblast, the networks were quickly contracted into small aggregates, similar to what is observed in MSC and fibroblast monocultures. No network structure can still be recognized at 24 hours. In contrast although Pl-Prc formed similar aggregates in monoculture, they did not show the same behavior in co-culture with EC. This indicates that the interaction between pericytes and endothelial cells has an effect on pericyte behavior.

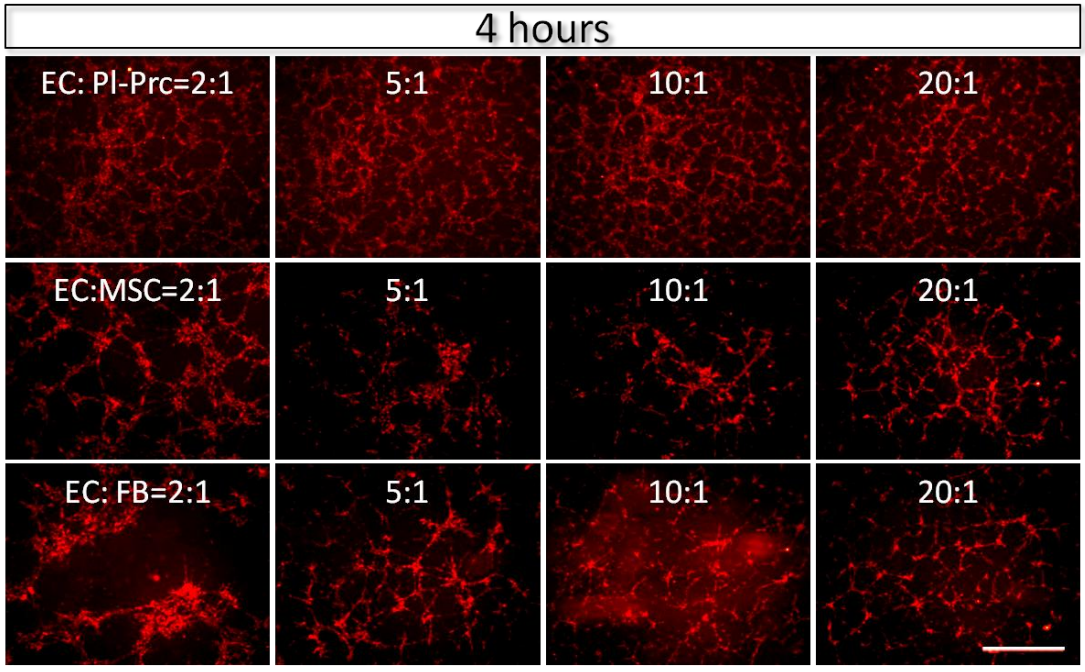



**Figure 16. Pl-Prc/ MSC/ FB co-culture with EC on Matrigel™ 4 hours after seeding.** EC were seeded in co-culture with Pl-Prc, MSCs, and fibroblasts at various ratios, respectively. EC were seeded at 30,000 cells per $cm^2$ on Matrigel™. Photos were taken at 4 hours after seeding. Only EC are shown here which were labeled with red fluorescent dye. Scale bar represents 1mm.

At four hours after seeding, for EC and Pl-Prc co-culture, there is no clear difference between networks containing different concentrations of Pl-Prc. Furthermore, the

networks appeared to be more structured compared to the network at four hours when EC is seeded alone (Figure 13).

For MSCs and fibroblasts, the cells rapidly assemble. At higher concentrations, EC can be seen to be contracted together, leaving only blank spaces behind.

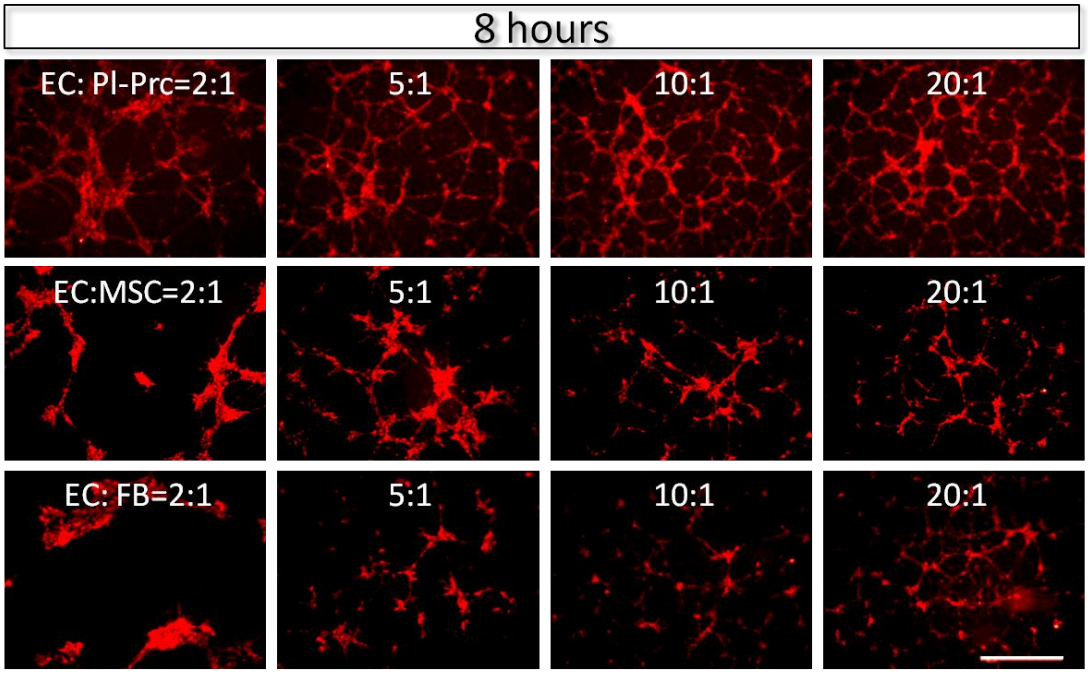



**Figure 17. Pl-Prc/ MSC/ FB co-culture with EC on Matrigel™ 8 hours after seeding** EC were seeded in co-culture with Pl-Prc, MSCs, and fibroblasts at various ratios, respectively. EC were seeded at 30,000 cells per $cm^2$ on Matrigel™. Photos were taken at 8 hours after seeding. Only EC are shown here which were labeled with red fluorescent dye.Scale bar represents 1mm.

At eight hours after seeding, for EC in co-culture with Pr-Plc, the tubes previously formed on Matrigel™ have completely taken shape. The morphology of the networks depended on the seeding density of Pl-Prc. At high Pl-Prc cell density, the network consisted of fewer and thicker tubes, while at low Pl-Prc densities, there were greater number of tubes in the networks, which resembled those in EC mono-culture on Matrigel™.

For EC in co-culture with MSCs and fibroblasts, the tubes were destroyed completely at high MSC or fibroblast density and only large aggregates of cells remained. At lower densities, the tubular network was only partially present and small cell aggregates and fragments with discontinuous tubes appeared.

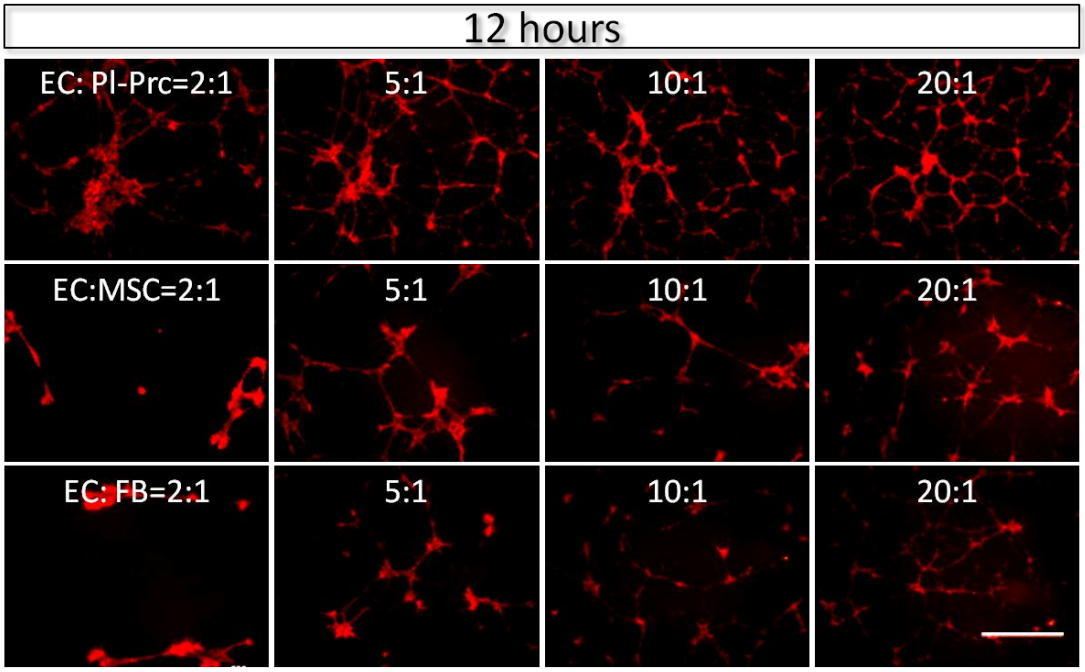



**Figure 18. Pl-Prc/ MSC/ FB co-culture with EC on Matrigel ™ 12 hours after seeding** EC were seeded in co-culture with Pl-Prc, MSCs, and fibroblasts at various ratios, respectively. EC were seeded at 30,000 cells per $cm^2$ on Matrigel™. Photos were taken at 12 hours after seeding. Only EC are shown here which were labeled with red fluorescent dye. Scale bar represents 1mm

At twelve hours after seeding, the tubes in networks of EC-Pl-Prc co-culture started to become less in number but thicker. At this point no break-down of the networks or the formation of aggregates was visible. The tubular structure at EC: Pl-Prc ratio 20:1 appeared similar to control where EC is seeded alone after 12 hours.

For MSCs and fibroblasts, almost all cells have contracted together and overall no network structure was present.

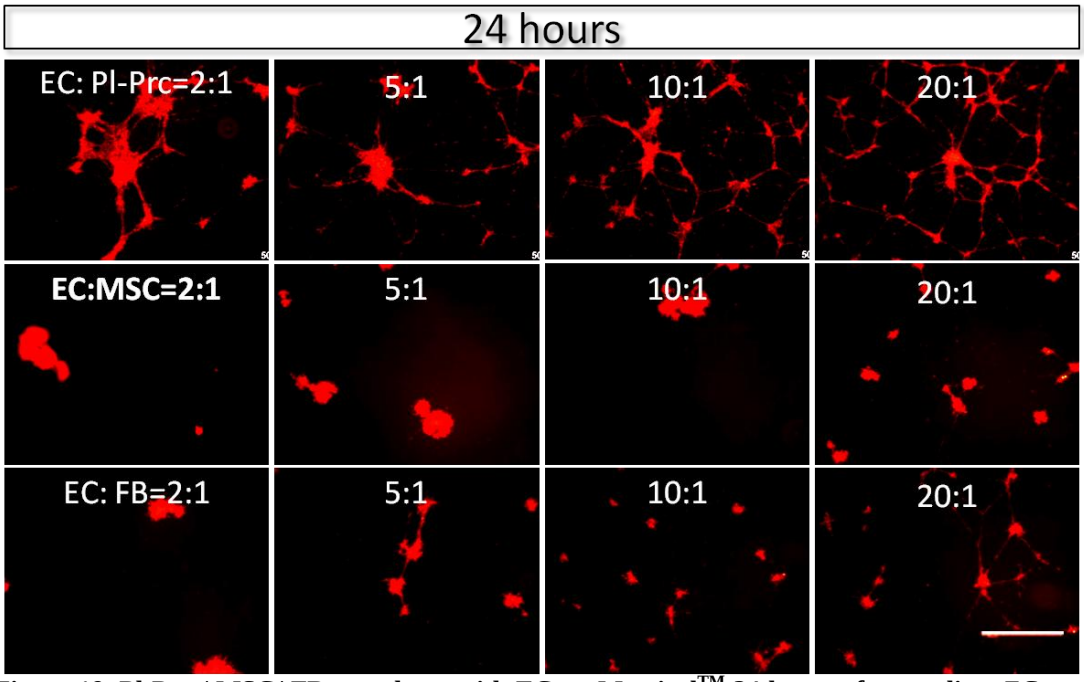



**Figure 19. Pl-Prc / MSC/ FB co-culture with EC on Matrigel™ 24 hours after seeding.** EC were seeded in co-culture with Pl-Prc, MSCs, and fibroblasts at various ratios, respectively. EC were seeded at 30,000 cells per $cm^2$ on Matrigel™. Photos were taken at 24 hours after seeding. Only EC are shown here which were labeled with red fluorescent dye. Scale bar represents 1mm

At 24 hours, the lower EC:Pl-Prc ratios 2:1 and 5:1 showed the formation of the first cell aggregates. For higher ratios 10:1 and 20:1 tubes have further combined to form less but thicker ones. In contrast to EC controls, less detached single cells which came from the break-down of the tubes was observed in the culture.

For MSCs and fibroblasts, except for EC-MSC 20:1 ratio all cells have formed aggregates and no more tubular structure was seen, even for low cell density samples.

# *5. Discussion*

This article sets out to test the hypothesis that pericytes are not only MSCs, but further possess properties that average MSCs are not able to substitute for. To this end, this study also aims to establish a set of *in vitro* assays that would be able to distinguish pericytes from other non-pericytic mesenchymal lineages such as MSCs and fibroblasts.

The findings revealed shortcomings of relying solely on the marker profile for pericyte identification, and also presented new evidences for the importance of functional assays to delineate the complet phenotype of pericytes. This thesis throws light especially on the following aspects:

## 5.1. The expression of MSC marker profile is not sufficient for distinguishing Pl-Prc, MSCs, and fibroblasts. Differentiation assay shows that Pl-Prc possess multi-potent differentiation potential as MSCs do.

The three mesenchymal cell types tested all expressed MSC hallmark markers and lack EC specific and hematopoietic markers, therefore meeting the marker expression criteria for being MSCs. However as fibroblasts are not MSCs. This proves that a MSC marker profile is necessary but not sufficient for confirming MSC identity of a cell population. A further criterion of defining MSC is their potential to differentiate into multiple mesenchymal lineages. As expected only pericytes and MSCs were able to give rise to osteoblasts, adipocytes, and chondrocytes, confirming their multipotency *in vitro*. Therefore pericytes behave like MSC in terms of multi-lineage differentiation.

Surprisingly, from the flow cytometry result for an extensive MSC marker panel, it is almost impossible to distinguish between Pl-Prc, MSCs, and the negative control fibroblasts. Therefore, the expression of MSC-related markers is necessary but not sufficient to identify MSCs.

Among the MSC-related antigens, CD146 in particular is worth noting. It is often used alone or in combination with other pericyte-related markers for sorting of pericytes from a heterogeneous population (Péault, et al., 2007; Crisan, et al., 2008; Covas, et al., 2008; PromoCell). However, the flow cytometry results show that Pl-Prc, MSCs, and fibroblasts all have similar level of expression for these two markers. It pose question on the efficacy of using CD146in identifying pericytes from other cell types *in vitro*, especially from MSCs and fibroblasts.

CD146, which is expressed in MSCs, ECs, and pericytes, is extensively used for pericyte identification and isolation (Péault, et al., 2007; Crisan, et al., 2008; Covas, et al., 2008). In fact the Pl-Prc used in this study were also isolated for CD146 (PromoCell). In this study, Pl-Prc, MSCs, and fibroblasts all exhibited high levels of CD146 expression. Therefore we conclude that CD146 is not specific enough to distinguish these closely related cell types. Cell sorting using CD146 as the sole marker may result in a heterogeneous population rather than pure pericytes.

Furthermore, the expression of CD146 is dynamic and passage dependent. We have conducted flow cytometry for later passages of Pl-Prc up to p+5 (p7), and we found that CD146 expression is significantly decreased with passaging. It is possible that CD146 is a dynamic marker and is gradually lost during cell passaging.

Therefore, although CD146 has been useful for isolating pericytes from tissues, it may not be a specific and stable marker for identification or purification of pericytes *in vitro*.

An interesting observation for differentiation assay is that fibroblasts showed the ability to produced calcium deposit after induction. However, as they are not able to produce fat droplets under adipogenic induction conditions they are not multipotent and can be clearly distinguished from MSC and pericytes. The ability to deposit calcium under osteogenic induction conditions is well known and was shown not to resemble the differentiation into osteoblasts (Ducy, et al., 2000; Cho, et al., 1992; Querido, et al., 2012).

It has been proposed that fibroblasts are very similar to differentiated osteoblasts. Their morphology in cell culture is difficult to distinguish. Furthermore, all genes that fibroblasts express are equally expressed in osteoblasts. However, it has also been point out that osteoblast, and not fibroblast, deposited minerized matrix outside the cell during cell culture (Ducy, et al., 2000). Cho and colleagues (1992) have shown that periodontal ligament fibroblasts, when induced with dexamethasone and ascorbic acid, are able to form mineralized matrix containing calcium deposit in immature form of hydroxyapatite. They have equally shown that these fibroblasts did not differentiate into osteoblast, and the morphology is different from mineralization of multi-potent stem cells in culture: the fibroblast cell body was elongated and they produced needle shaped crystals with highly aligned fibers, and did not resemble the bone matrix formed in vivo. On the other hand, cells that undergo typical osteogenic differentiation show minerized nodules that resembles real bone tissues, with densely

mineralized centers, and less minerized surrounding regions. The collagen fibers in the bone matrix are poorly oriented, with globular mineral deposits (Querido, et al., 2012). In our hands, only cultures of pericytes and MSC showed calcified centers resembling nodules, which expanded as the differentiation assay. Fibroblast showed rather strongly calcified areas with sharp edges and even Ca2+ distribution, therefore lacking the expanding nodules. (Figure 7) Therefore we conclude that fibroblast rather did not differentiate into osteoblast, but this will have to be confirmed further.

It has been noted that the lipid droplets in Pl-Prc are smaller in size compared to those in MSCs, while the number of cells that produced lipid droplets are greater for Pl-Prc. However, it is possible that since the two cell types come from different tissue of origin, their morphology turned out not exactly the same. In fact, it has been reported that among MSCs from different tissues, the adipogenic differentiation outcomes were different. MSCs derived from umbilical cord blood, for example, gives tiny lipid droplets compared to MSCs from bone marrow or adipose tissue (Rebelatto, et al., 2008).

To conclude, both the MSC-related marker profile as well as *in vitro* differentiation assay are not able to distinguish between a typical pericyte population (Pl-Prc) and a typical bone marrow MSC population. This is in agreement with current literature where CD146-isolated pericytes from different tissues have been shown to display MSC features. However, we went one step further to test if Pl-Prc and MSC share not only MSC-related characteristics, but also pericytic characteristics.

## 5.2. NG2, desmin and Tie2 may serve as pericyte-specific markers

The expression of two conventional pericyte markers, α-SMA and PDGFR-β, were not able to distinguish pericytes from MSCs in this study. The level of α-SMA expression in Pl-Prc is actually lower than that in MSC. These two markers are equally expressed in fibroblasts. Therefore, they are not really pericyte specific for *in vitro* culture.

NG2, desmin and Tie2, on the other hand, showed an unambiguously stronger expression in Pl-Prc than in MSCs and fibroblast. NG2 and desmin are well known pericyte markers (reviewed by Díaz-Flores, et al., 2009), while Tie2 is a novel antigen that we have discovered to be potentially pericyte-specific.

These three markers are the first pieces of evidence that there may be some differences between MSCs and Pl-Prc. MSCs and fibroblasts expressed none of the three markers, indicating that they may lack some pericyte features.

While the exact role of NG2 is still unclear, desmin and Tie2 are possibly involved in pericyte function. Desmin is a contractile protein and is regarded as a late pericyte marker (Song, 2005). The other marker, Tie2, showed the potential of being able to distinguish pericytes from other cells from the mesenchymal lineage. Moreover, Tie2 has also important functions in angiogenesis. Ang1/Tie2 is one of ligand/ receptor pairs that form the bases of EC-pericyte interaction (Suri, et al., 1996). Tie2 has been reported to be expressed by retina pericytes (Cai, et al., 2008), however it has not been employed for pericyte identification or isolation so far. As CD146 is not

selective for pericytes among other mesenchymal cells, Tie2 represents a promising candidate for future pericyte identification and isolation. However, further validation of Tie2 expression in pericytes will be needed.

Another valuable piece of information from ICC is that the staining pattern of α-SMA, PDGFR-β, and NG2 in Pl-Prc suggest that pericytes in this study were not fully activated. For α-SMA, Pl-Prc did not show staining in the form of α-SMA fibers as in the case of MSCs, which indicates that α-SMA has not yet assemble into functional form, even the protein is present in the cell. With regards to PDGFR-β, and NG2, it is worth noting that although both antigens are surface markers, the expression of these markers in Pl-Prc was not on the cell surface, but rather very possibly still at the endoplasmic reticulum or the Golgi apparatus. These two proteins have not been transferred to the cell surface and were thus not yet functional. It has been reported that pericyte expression of α-SMA is up-regulated in proliferating microbasculatures, and its in vitro expression can be up-regulated by transforming growth factor β1 (TGF-β1 ) (Verbeek, et al., 1994) or by removing fibroblast growth factor 2 (FGF-2) (Papetti, et al., 2003). It is possible that after being extracted from its native niche in placenta tissue, the pericyte cells have suspended some of their original functions and were in a "quiescent state".

Together with the pattern of CD146 expression, the results suggest also that the marker expression profile of pericytes is dynamic and may depend on the culturing and development state of the cells. Moreover, the marker expression for pericytes in *in vitro* culture may be significantly different from that in *in vivo*.

## 5.3. EC-network maintenance, not co-localization, is characteristic of pericytes

The capability of pericytes to co-localize with EC networks on Matrigel™ is often cited to be a pericyte-related characteristic. Song et al. (2005) used the combination of PDGFR-β/desmin/NG2/α-SMA expression and co-localization with EC networks on Matrigel as the criteria to judge if perivascular cells are pericytes. Darland et al. (2001) characterized MSC's differentiation into pericytes through their NG2/α-SMA expression and their co-localization and network formation capability when co-cultured with EC. However, we propose that co-localization with EC network on Matrigel™ is not a pericyte-exclusive feature. By co-culturing EC with Pl-Prc, MSCs, or fibroblasts, we showed that all the three cell types were able to co-localize with EC-formed networks, and there is no observable difference in the way in which the cells distribute themselves. The behavior of co-localization with EC-formed vessels on Matrigel™ is thus not sufficient for identifying pericyte *in vitro*.

When the co-culture is maintained for a longer period of time, the difference in the extent that the network is preserved is clear. Only Pl-Prc at low cell density managed to maintain most of the network structure for up to 24 hours, while MSCs and fibroblast contracted it significantly so that no proper network could be identified anymore.

## 5.4. A bold guess: MSCs may be pericytes that have partially lost their pro-angiogenesis potential

Despite the clear differences in their pro-angiogenic capacity, MSCs apparently share a large number of features with Pl-Prc. It indicates that MSCs and Pl-Prc are closely related populations. It is possible that both cells come from a common progenitor. Alternatively, one may have derived from another.

We have shown that during culture, pericytes gradually lose some of their marker expression. It is natural to suppose that the pro-angiogenic capacity as well as the pericytic markers in MSC have been lost during development.

It is equally possible that these features were lost due to different isolation and culturing methods, since pericytes are highly sensitive to the microenvironment that they are exposed to.

However, this hypothesis still needs to be validated further.

# *6. Conclusion*

This study has compared placenta pericytes, bone marrow derived MSCs, and fibroblasts in terms of the MSC-related features and pericytic features.

Complementary to current literature, this study show that Pl-Prc are *bona fide* MSCs for their expression of MSC-related antigen panel, and their ability to differentiate into different lineages. At the same time, it shows the novel observation that MSCs do not express the pericyte related markers NG2, desmin, and Tie2.

Furthermore, we came up with a new functional assay on Matrigel$^{TM}$ that is sensitive enough to distinguish Pl-Prc from MSCs and fibroblasts. Although the short term effects of EC-Pl-Prc/MSC/fibroblasts are similar, a prolonged co-culture system showed that only Pl-Prc has the capacity to stabilized EC-formed network over an extended period of time, confirming their superior pro-angiogenic capacity compared to non-pericytic mesenchymal populations.

Therefore, by comparing different cell populations using both marker expression and functional assays. we conclude that MSC markers expression and differentiation assay alone are not sufficient to distinguish pericyte from other mesenchymal populations. Pl-Prc do possess specific marker expression (NG2, desmin, and Tie2) and pro-angiogenic functions that MSCs do not share.

This new Matrigel$^{TM}$ stabilization assay may serve as a useful tool for screening pericytes *in vitro*. This assay can be used to complement the marker characterization of pericytes, which may be dynamic and may depend on culturing conditions. This

model directly accesses the cells' ability to interact with and to maintain EC-formed networks on Matrigel$^{TM}$, which is the fundamental function of pericytes.

The major limitation of this study is the cell sources used. Commercial cells were used throughout this study as representative populations of Pl-Prc and MSC. Although both populations were isolated and maintained using state-of –the-art protocols, several limitation may still apply. First, although CD146+CD105+CD45-CD34- population from human placenta has been shown to be exclusively pericytes (Crisan, et al., 2008), the cells used were never tested for their in situ location in their tissue of origin. Secondly, as the pericytes and MSCs used come from different sources, it is possible that some of the differences in their marker expression and angiogenic properties are actually attributed to their different tissues of origin. A vigorous study would necessitate comparison of pericytes and MSCs from the same cell source. Our group has demonstrated that pericytes and MSCs isolated from the same bone marrow sample demonstrated different pro-angiogenic potentials through the MatrigelTM assay (Blocki, et al., under review), thus further validated the sensitivity of the MatrigelTM stabilization assay, as well as pericytes' additional role in capillary maintenance.

# *Bibliography*

**Ankrum, J. and Karp, J. M. 2010.** Mesenchymal stem cell therapy: Two steps forward, one step back. *Trends Mol Med.* 2010, Vol. 16, 5, pp. 203-9.

**Armulik, Annika, Abramsson, Alexandra and Betsholtz, Christer. 2005.** Endothelial/pericyte interactions. *Circulational Research.* 2005, Vol. 97, 6, pp. 512-23.

**Armulik, Annika, Genove, Guillem and Betsholtz, and Christer. 2011.** Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Developmental Cell.* 2011, Vol. 21, 2, pp. 193-215.

**Augello, A., Kurth, T. B. and De Bari, C. 2010.** Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. *Eur Cell Mater.* 2010, Vol. 20, pp. 121-133.

**Barberi, T., et al. 2006.** Derivation of multipotent mesenchymal precursors from human embryonic stem cells. *PloS Med.* 2006, Vol. 2, 6.

**Barry, Frank, et al. 2001.** The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. *Biochemical and Biophysical Research Communications.* 2001, Vol. 289, 2, pp. 519-24.

**Bauvois, Brigitte and Dauzonne, Daniel. 2006.** Aminopeptidase-N/CD13(EC 3.4.11.2) Inhibitors:Chemistry, Biological Evaluations,andTherapeutic Prospects. *Med Res Rev.* 2006, Vol. 26, 1, pp. 88-130.

**BD. 2008.** *Manual: BD matrigel 354234.* s.l. : BD Bioscience, 2008.

**Bergers, G. 2008.** Pericytes, the mural cells of the microvascular system. [book auth.] W. D., Folkman, J. Figg. *Angiogenesis.* s.l. : Springer, 2008, pp. 45-5.

**Bergers, Gabriele and Song, Steven. 2005.** The role of pericytes in blood-vessel formation and maintenance. *Nero Oncol.* 2005, Vol. 7, 4.

**Bergwerff, Maarten, et al. 1998.** Neural crest cell contribution to the developing circulatory system: implications for vascular morphology? *Circulation Research.* 1998, Vol. 82, 2, pp. 221-31.

**Bianco, P., et al. 1988.** Alkaline phosphatase positive precursors of adipocytes in the human bone marrow. *Br J Haematol.* 1988, Vol. 68, 4, pp. 401-3.

**Bianco, P., Robey, P. G. and Simmons, P. J. 2008.** Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell.* 2008, Vol. 2, 4, pp. 313-9.

**Bianco, Paolo. 2011.** Back to the future: moving beyond “Mesenchymal Stem Cells”. *J Cell Biochem.* 2011, Vol. 112, 7, pp. 1713-21.

**Bishop, Eileen T., et al. 1999.** An in vitro model of angiogenesis: basic features. *Angiogenesis.* 1999, Vol. 3, 4, pp. 335-44.

**Blocki, A., et al. under review.** Not all MSCs can be pericytes: Functional in vitro assays to distinguish pericytes from other mesenchymal stem cells in angiogenesis. *Stem Cell Development.* under review.

**Bonab, Mandana Mohyeddin, et al. 2006.** Aging of mesenchymal stem cell in vitro. *BMC Cell Biol. .* 2006, Vol. 7, 14.

**Bouacida, A., et al. 2012.** Pericyte-like progenitors show high immaturity and engraftment potential as compared with mesenchymal stem cells. *PLoS One.* 2012, Vol. 7, 11.

**Cai, J, et al. 2008.** The angiopoietin/Tie-2 system regulates pericyte survival and recruitment in diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2008, Vol. 49, 5, pp. 2163-71.

**Cai, Jun, et al. 2008.** he Angiopoietin/Tie-2 System Regulates Pericyte Survival and Recruitment in Diabetic Retinopathy. *IOVS.* 2008, Vol. 49, 5.

**Caplan, A. I. 2007.** Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *Journal of Cellular Physiology.* 2007, 213, pp. 341-7.

**Caplan, A. I. 2008.** All MSCs are pericytes? *Cell Stem Cell.* 2008, Vol. 3, 3, pp. 229-30.

**Caplan, A. I. 1994.** The mesengenic process. *Clin Plast Surg.* 1994, Vol. 21, 3, pp. 429-35.

**Castrechini, N. M., et al. 2010.** Mesenchymal stem cells in human placental chorionic villi reside in a vascular niche. *Placenta.* 2010, Vol. 31, 3, pp. 203-12.

**Cho, Moon I., et al. 1992.** In vitro formation of mineralized nodules by periodontal ligament cells from the rat. *Calcif Tissue Int.* 1992, Vol. 50.

**Corselli, M., et al. 2010.** Perivascular ancestors of adult multipotent stem cells. *Arterioscler Thromb Vasc Biol.* 2010, Vol. 30, 6, pp. 1104-9.

**Corselli, M., et al. 2012.** The tunica adventitia of human arteries and veins as a source of mesenchymal stem cells. *Stem Cells Dev.* 2012, Vol. 21, 8, pp. 1299-308.

**Covas, Dimas T., et al. 2008.** Multipotent mesenchymal stromal cells obtainedfrom diverse human tissues share functional propertiesand gene-expression profile with CD146+ perivascular cells and fibroblasts. *Experimental hematology.* 2008, Vol. 36, 5, pp. 641-54.

**Craig, W., et al. 1993.** Expression of Thy-1 on human hematopoietic progenitor cells. 1993, Vol. 177, 5, pp. 1331-42.

**Crisan, M., et al. 2008.** A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell.* 2008 йил, Vol. 3, 3, pp. 301-13.

**Crisan, Mihaela, et al. in press.** Perivascular cells for regenerative medicine. *J Cell Mol Med.* in press.

**da Silva Meirelles, L., Chagastelles, P. C. and Nardi, N. B. 2006.** Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science.* 2006, Vol. 119, Pt 11, pp. 2204-13.

**Darland, D. C., et al. 2003.** Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. *Dev Biol.* 2003, Vol. 264, 1, pp. 275-88.

**Darland, D.C. and D'Amore, P.A. 2001.** TGF beta is required for the formation of capillary-like structures in three-dimensional co-cultures of 10T1/2 and endothelial cells. *Angiogenesis.* 2001, Vol. 4, 1, pp. 11-20.

**Dellavalle, A, et al. 2007.** Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat Cell Biol.* 2007, Vol. 9, 3, pp. 255-67.

**Díaz-Flores, L., et al. 2009.** Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. *Histol Histopathol.* 2009, Vol. 24, 7, pp. 909-69.

**Dominici, M, et al. 2006.** Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006, Vol. 8, 4, pp. 315-7.

**Donovan, D., et al. 2001.** Comparison of three in vitro human 'angiogenesis' assays with capillaries formed in vivo. *Angiogenesis.* 2001, Vol. 4, 2, pp. 113-21.

**Ducy, Patricia, Schinke, Thorsten and Karsenty, Gerard. 2000.** The osteoblast: a sophisticated fibroblast under central surveillance. *Science.* 2000, Vol. 289, 1501.

**Dumont, DJ, et al. 1992.** tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. *Oncogene.* 1992, Vol. 7, 8.

**Escribano, Luis, et al. 1998.** Expression of the c-kit (CD117) Molecule in Normaland Malignant Hematopoiesis. *Keukemia and Lymphoma.* 1998, Vol. 30, 5-6, pp. 459-466.

**Farrington-Rock, C., et al. 2004.** Chondrogenic and adipogenic potential of microvascular pericytes. *Circulation.* 2004, Vol. 110, 15, pp. 2226-32.

**Ferrara, N, Gerber, HP and LeCouter, J. 2003.** The biology of VEGF and its receptors. *Nat Med.* 2003, Vol. 9, 6, pp. 669-76.

**Folkman, Judah and Haudenschild, Christian. 1980.** Angiogenesis in vivo. *Nature.* 1980, Vol. 288, 5791, pp. 551-6.

**Fonsatti, Ester, et al. 2001.** Endoglin: An accessory component of the TGF-beta-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. *Journal of Cellular Physiology.* 2001, Vol. 188, 1, pp. 1-7.

**Francis, S. E., et al. 2002.** Central roles of alpha5beta1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. *Arterioscler Thromb Vasc Biol.* 2002, Vol. 22, 6, pp. 927-33.

**Franco, M., et al. 2011.** Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression. *Blood.* 2011, Vol. 118, 10, pp. 2906-17.

**Friedenstein, A. J., Chailakhjan, R. K. and Lalykina, K. S. 1970.** The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet.* 1970, Vol. 3, 4, pp. 393-403.

**Friedenstein, A. J., et al. 1968.** Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation.* 1968, Vol. 6, 2, pp. 230-47.

**Gaengel, K., et al. 2009.** Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler Thromb Vasc Biol.* 2009 йил, Vol. 29, 5, pp. 630-638.

**Gerhardt, Holger and Betsholtz, Christer. 2003.** Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res.* 2003, Vol. 314, 1, pp. 15-23.

**Gerson, Stanton L. 1999.** Mesenchymal stem cells: No longer second class marrow citizens. *Nature Medicine.* 1999, Vol. 5, 3, pp. 262-4.

**Haeryfar, S. M. Mansour and Hoskin, David W. 2004.** Thy-1: more than a mouse pan-T cell marker. *The Journal of Immunology.* 2004, Vol. 103, 6, pp. 3581-8.

**Hellström, M., et al. 1999.** Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development.* 1999, Vol. 126, 14, pp. 3047-55.

**Hellström, Mats, et al. 2001.** Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *The Journal of Cell Biol.* 2001, Vol. 153, 3.

**Hermiston, ML, Xu, Z and Weiss, A. 2003.** CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol.* 2003, Vol. 21, pp. 107-37.

**Hinz, Boris, et al. 2001.** Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell.* 2001, Vol. 12, 9, pp. 2730-41.

**Hirschi, K. K., Rohovsky, S. A. and D'Amore, P. A. 1998.** PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol.* 1998, Vol. 141, 3, pp. 805-14.

**Hirschi, Karen K. and Amore, Patricia A. D. 1996.** Pericyte in the microvasculature. *Cardiovascular Research.* 1996, Vol. 32, 4, pp. 687-98.

**Holmes, Katherine, et al. 2007.** Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. *Cell Signal.* 2007, Vol. 19, 10, pp. 2003-12.

**Hynes, R. O. 2002.** Integrins: bidirectional, allosteric signaling machines. *Cell.* 2002, Vol. 110, 6, pp. 673-87.

**Hynes, R. O. 1992.** Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 1992, Vol. 69, 1, pp. 11-25.

**Ip, James E., et al. 2007.** Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. *Mol Biol Cell.* 2007, Vol. 18, 8, pp. 2873-82.

**Jiang, Yuehua, et al. 2002.** Pluripotency of mesenchymal stem cellsderived from adult marrow. *Nature.* 2002.

**Kleinman, Hynda K. and Martin, George R. 2005.** Matrigel: Basement membrane matrix with biological activity. *Semin Cancer Biol.* 2005, Vol. 15, 5, pp. 378-86.

**Koh, Wonshill, et al. 2008.** In vitro three dimensional collagen matrix models of endothelial lumen formation during vasculogenesis and angiogenesis. *Methods in Ensymology.* 2008, Vol. 443, pp. 83-101.

**Kolf, Catherine M, Cho, Elizabeth and Tuan, Rocky S. 2007.** Biology of adult mesenchymal stem cells:regulation of niche, self-renewal and differentiation. *Arthritis Research & Therapy.* 2007, Vol. 9, 1, pp. 1196-2116.

**Korff, T, et al. 2001.** Blood vessel maturation in a 3-dimensional spheroidal co-culture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB.* 2001, Vol. 15, 2, pp. 447-57.

**Kurz, Haymo, et al. 2008.** Pericytes in the mature chorioallantoic membrane capillary plexus contain desmin and α-smooth muscle actin: relevance for non-sprouting angiogenesis. *Histochem Cell Biol.* 2008, Vol. 130, 5, pp. 1027-40.

**Lamagna, Chrystelle and Bergers, Gariele. 2006.** The bone marrow constitutes a reservoir of pericyte progenitors. *J Leukoc Biol.* 2006, Vol. 80, 4, pp. 677-81.

**Levée, Per, et al. 1994.** Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes & Dev.* 1994, Vol. 8, 16, pp. 1875-87.

**Li, Longxuan, et al. 2012.** An angiogenic role for the α5β1 integrin in promoting endothelial cell proliferation during cerebral hypoxia. *Experimental Neurology.* 2012, Vol. 237, 1, pp. 46-54.

**Li, Zhenlin and Paulin, Denise. 1991.** High level desmin expression depends on a muscle-specific enhancer. *The Journal of Biological Chemistry.* 1991, Vol. 266, 10, pp. 6562-70.

**Lonza. 2011.** *Poietics™ hMSC human mesenchymal stem cells & media .* Walkersville : Lonza, 2011.

**Mazzone, A and Ricevuti, G. 1995.** Leukocyte CD11/CD18 integrins: biological and clinical relevance. *Haematologica.* 1995, Vol. 80, 2, pp. 161-75.

**McDonald, D. M. 2008.** Angiogenesis and vascular remodeling in inflammation and cancer: biology and architecture of the vasculature. [book auth.] W. D., Folkman, J. Figg. *Angiogenesis.* s.l. : Springer, 2008, p. 17.

**McDonald, Donald M and Choyke, Peter L. 2003.** Imaging of angiogenesis: from microscope to clinic. *Nature Medicine.* 2003, Vol. 9, 6, pp. 713-25.

**McFarland, H. I., et al. 1992.** CD11b (Mac-1): a marker for CD8+ cytotoxic T cell activation and memory in virus infection. *The Journal of Immunology.* 1992, Vol. 149, 4, pp. 1326-33.

**Morgensterna, Daniel A, et al. 2003.** Expression and glycanation of the NG2 proteoglycan in developing, adult, and damaged peripheral nerve. *Molecular and Cellular Neuroscience.* 2003, Vol. 24, 3, pp. 787-802.

**Nakano, Akinobu, et al. 2008.** Expression of leukocyte common antigen (CD45) on various human leukemia/lymphoma cell lines. *Acta Pathol Jpn.* 2008, Vol. 40, 2, pp. 107-15.

**Nauta, A. J. and Fibbe, W. E. 2007.** Immunomodulatory properties of mesenchymal stromal cells. *Blood.* 2007, Vol. 110, 10, pp. 3499-506.

**Nehls, Volker and Drenckhahn, Detlev. 1991.** Heterogeneity of microvascular pericytes for smooth muscle type α-actin. *The Journal of Cell Biology.* 1991, Vol. 113, 1, pp. 147-54.

**Nehls, Volker, Denzer, Kristin and Drenckhahn, Detlev. 1992.** Pericyte involvement in capillary sprouting during angiogenesis in situ. *Cells & Tissue Research.* 1992, Vol. 270, 3.

**Nielsen, Julie S. and McNagny, Kelly M. 2008.** Novel functions of the CD34 family. *J Cell Sci.* 2008, Vol. 121, Pt 22, pp. 3683-92.

**Nombela-Arrieta, César, Ritz, Jerome and Silberstein, Leslie E. 2011.** The elusive nature and function of mesenchymal stem cells. *Nat Rev Mol Cell Biol.* 2011, Vol. 12, 2, pp. 126-31.

**Oczenski, Wolfgang, et al. 2003.** HLA-DR as a marker for inscreased risk for systemic inflammation and septic complications after cardiac surgery. *Intensive Care Med.* 2003, Vol. 29, 8, pp. 1253-7.

**Ohneda, Osamu, et al. 2001.** ALCAM (CD166): its role in hematopoietic and endothelial development. *Blood.* 2001, Vol. 98, 7, pp. 2134-42.

**Orlidge, Alicia and D'Amore, Patricia A. 1987.** Inhibition of Capillary Endothelial Cell Growthby Pericytes and Smooth Muscle Cells. *The Journal of Cell Biology.* 1987, Vol. 105, 3, pp. 1455-62.

**Ozerdem, U., et al. 2005.** Contribution of bone marrow-derived pericyte precursor cells to corneal vasculogenesis. *Invest Ophthalmol Vis Sci.* 2005, Vol. 46, 10, pp. 3502-6.

**Ozerdem, Ugur and Stallcup, William B. 2003.** Early contribution of pericytes to angiogenic sprouting and tube formation. *Angiogenesis.* 2003, Vol. 6, 3, pp. 241-9.

**Ozerdem, Ugur and Stallcup, William B. 2004.** Pathological angiogenesis is reduced by targeting pericytes via the NG2 proteoglycan. *Angiogenesis.* 2004, Vol. 7, 3, pp. 269-76.

**Ozerdem, Ugur, et al. 2001.** NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. *Dev Dyn.* 2001, Vol. 222, 2, pp. 218-27.

**Papetti, M., et al. 2003.** Fibroblast growth factor represses Smad-mediated myofibroblast activation in aortic valvular interstitial cells. *Invest Ophthalmol Vis Sci.* 2003, Vol. 44, 11, pp. 4994-5005.

**Péault, Bruno, et al. 2007.** Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. *Molecular Therapy.* 2007, Vol. 15, 5, pp. 867-77.

**Pittenger, Mark F., et al. 1999.** Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999, Vol. 284, 5411, pp. 143-7.

**Pont, Suzanne. 1987.** Thy-1: a lymphoid cell subset marker capable of delivering an activation signal to mouse T lymphocytes. *Biochemie.* 1987, Vol. 69, pp. 315-20.

**PromoCell.** Pericytes. [Online] [Cited: May 28, 2012.] http://www.promocell.com/fileadmin/promocell/PDF/C-12980.pdf.

**Promocell.** *Pericytes Instruction Manual.* Heidelberg : PromoCell.

**Puri, M C, et al. 1995.** The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO.* 1995, Vol. 14, 23, pp. 5884-91.

**Puri, Mira C. and Bernstein, Alan. 2003.** Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis. *PNAS.* 2003, Vol. 100, 22.

**Querido, W, Farina, M and Balduino, A. 2012.** Giemsa as a fluorescent dye for mineralizing bone-like nodules in vitro. *Biomed. Mater.* 2012, Vol. 7, 1.

**Rajantie, I., et al. 2004.** Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. *lood.* 2004, Vol. 104, 7, pp. 2084-6.

**Rajkumar, V. S., et al. 2006.** Platelet-derived growth factor-beta receptor activation is essential for fibroblast and pericyte recruitment during cutaneous wound healing. *Am J Pathol.* 2006 йил, Vol. 169, 6, pp. 2245-65.

**Rebelatto, C. K., et al. 2008.** Dissimilar Differentiation of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, and Adipose Tissue. *Exp. Biol. Med.* 2008, Vol. 223, 7.

**Rege, Tanya A. and Hagood, James S. 2006.** Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *The FASEB Journal.* 2006, Vol. 20, 8, pp. 1045-54.

**Resta, Regina, Yamashita, Yoshio and Thompson, Linda F. 1998.** Ecto-enzyme and signaling functions of lymphocyte CD73. *Immunol Rev.* 1998, Vol. 161, pp. 95-109.

**Rucker, HK, Wynder, HJ and Thomas, WE. 2000.** Cellular mechanisms of CNS pericytes. *Brain Res Bull.* 2000, Vol. 51, 5.

**Sacchetti, B., et al. 2007.** Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell.* 2007, Vol. 131, 2, pp. 324-36.

**Salasznyk, R. M., et al. 2004.** Adhesion to vitronectin and collagen I promotes osteogenic differentiation of human mesenchymal stem cells. *J Biomed Biotechnol.* 2004, Vol. 2004, 1, pp. 24-34.

**Schlingemann, Reinier O., et al. 1990.** Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. *American Journal of Pathology.* 1990, Vol. 136, 6, pp. 1393-405.

**Schoolmeesters, A., et al. 2009.** Functional profiling reveals critical role for miRNA in differentiation of human mesenchymal stem cells. *PLoS One.* 2009, Vol. 4, 5, p. e5605.

**Senger, D. R., et al. 1996.** Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative

mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. *Am J Pathol.* 1996, Vol. 149, 1, pp. 293-305.

**Shi, Songtao and Gronthos, Stan. 2003.** Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res.* 2003, Vol. 18, 4, pp. 696-704.

**Shih, Ie-Ming, et al. 1994.** Melanoma-associated Antigen Isolation and Functional Characterization of the A32. *Cancer Res.* 1994, Vol. 54, 9, pp. 2514-20.

**Sims, David E. 1986.** The pericyte --a review. *Tissue &Cell.* 1986, Vol. 18, 2, pp. 153-74.

**Song, S., Ewald, A. J., Stallcup, W., Werb, Z., Bergers, G. 2005.** PDGF-beta+ perivascular progenitor cells in tumors regulate pericyte differentiation and vascular survival. *Nat Cell Biol.* 2005 йил, Vol. 7, 9, pp. 870-879.

**Soriano, Philippe. 1994.** Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes & Dev.* 1994, Vol. 8, 16, pp. 1888-96.

**Springer, Timothy, et al. 1979.** Mac-1: a macrophage differentiation antigen identifiedby monoclonal antibody. *Eur. J. Immunol.* 1979, Vol. 9, 4, pp. 301-6.

**Suri, Chitra, et al. 1996.** Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell.* 1996, Vol. 87, 7, pp. 1171-80.

**Tedder, Thomas F. 2009.** CD19: a promising B cell target for rheumatoid arthritis. *Nat Rev Rheumatol.* 2009, Vol. 5, 10, pp. 572-7.

**U. S. National Institutes of Health. 2012.** [Online] 2012. [Cited: December 6, 2012.] http://clinicalTrials.gov.

**Ucuzian, Areck A. and Greisler, Howard P. 2007.** In vitro models of angiogenesis. *World Journal of Surgery.* 2007, Vol. 31, 4, pp. 654-63.

**Verbeek, M. M., et al. 1994.** Induction of alpha-smooth muscle actin expression in cultured human brain pericytes by transforming growth factor-beta 1. *Am J Pathol.* 1994, Vol. 144, 2, pp. 372-82.

**Verhoeven, D and Buyssens, N. 1988.** Desmin-positive stellate cells associated with angiogenesis in a tumour and non-tumour system. *Virchows Arch B Cell Pathol Incl Mol Pathol.* 1988, Vol. 54, 5, pp. 263-72.

**Vestweber, Dietmar. 2008.** VE-Cadherin, the major endothelial adhesion moleculre controlling cellular junctions and blood vessel formation. *Arterioscler Thromb Vasc Biol.* 2008, Vol. 28, 2, pp. 223-32.

**Wakui, Shin, et al. 2006.** Localization of Ang-1, -2, Tie-2, and VEGF expression at endothelial-pericyte interdigitation in rat angiogenesis. *Laboratory Investigation.* 2006, Vol. 86, 11, pp. 1172-84.

**Wickström, Malin, et al. 2011.** Aminopeptidase N (CD13) as a target for cancer chemotherapy. *Cancer Sci.* 2011, Vol. 102, 3, pp. 501-8.

**Yamashita, H., et al. 1994.** Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor-beta. *Journal of Biological Chemistry.* 1994, Vol. 269, 3, pp. 1995-2001.

**Yamashita, Jun, et al. 2000.** Flk1-positive cells derived fromembryonic stem cellsserve as vascular progenitors. *Nature.* 2000, Vol. 408, 6808, pp. 92-6.

**Yoshiike, T., et al. 1991.** HLA-DR antigen expression on peripheral T cell subsets in pityriasis rosea and herpes zoster. *Dermatologica.* 1991, Vol. 182, 3, pp. 160-3.

**Zannettino, A. C., et al. 2008.** Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *J Cell Physiol.* 2008, Vol. 214, 2, pp. 413-21.

**Zimmerlin, Ludovic, et al. 2009.** Stromal vascular progenitors in adult human adipose tissue. *Journal of the International Society for Advancement of Cytometry.* 2009, Vol. 77, 1, pp. 22-30.